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COMPOSITION AND STABILITY OF BIOACTIVE METABOLITES FOR BOTANICAL FOOD SUPPLEMENTS: THE CASE OF ROSEMARY AND PIGMENTED POTATOES

Settore Scientifico Disciplinare CHIM/10

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Ai miei genitori,

a Francesco

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INTRODUCTION

1. NUTRACEUTICALS

In recent years, we are seeing the evolution of a new diet paradigm which places more emphasis on the health benefits of diet itself. “Let food be thy medicine and medicine be thy food”, quoted by Hippocrates about 2,500 years ago is certainly the tenet of today.

Nutraceuticals are the emerging class of natural products that makes the line between food and drugs to fade. Although the use of nutraceuticals by people has a long history, only recent scientifically supported nutritional and medical evidence has allowed nutraceuticals to emerge as being potentially effective (Dillard & German, 2000).

Nutraceutical is a term coined from “nutrition” and “pharmaceutical” to describe substances which are not traditionally recognized nutrients (e.g. vitamins and minerals) but which have positive physiological effects on the human body. The term was originally used by Defelice in 1989 with the definition “A food or parts of food that provide medical or health benefits, including the prevention and/or treatment of a disease”. On the other hand, Health Canada defines nutraceutical as “a product prepared from foods, but sold in the form of pills, or powder (potions) or in other medicinal forms, not usually associated with foods” (Wildman, 2001; Bull, 2000).

Physiological effects of nutraceuticals do not easily fall into the legal categories of food or drug but inhabit a grey area between the two (Figure 1).

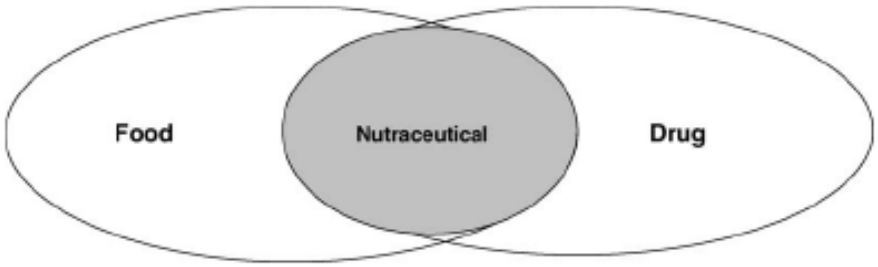


Figure 1. Nutraceutical occupies position between food and drug (Gulati & Ottaway, 2006)

Within European Union (EU) law the legal categorization of a nutraceutical is, in general, made on the basis of its accepted effects on the body.

These products, in general sense, cover health promotion, "optimal nutrition" and concept of enhanced performance – both physically and mentally – and reduction of disease risk factors.

They represent nutraceuticals, functional and fortified foods (Richardson, 1996). Broadly, functional and fortified foods are those with a similar appearance to their traditional counterparts, while nutraceuticals are components that are often consumed in unit dose forms such as tablets, capsules or liquids and commonly known as food/dietary supplements. The nutraceutical, functional and fortified food sectors have grown significantly in Europe during the last decade. In the global marketplace nutraceuticals and functional foods have become a multi-billion dollar industry and estimates within Canada suggest that the Canadian nutraceutical and functional food industry has potential to grow to $50 billion US.

Nutraceuticals covers most of the therapeutics areas such as anti-arthritic, cold and cough, sleeping disorders, digestion and prevention of certain cancers, osteoporosis, blood pressure, cholesterol control, pain killers, depression and diabetes (Figure 2) (Pandey *et al*., 2010).

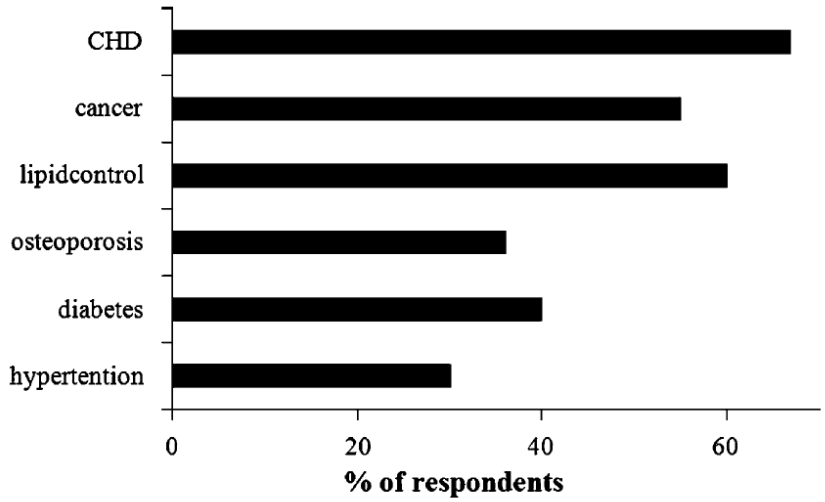


Figure 2. Therapeutic areas covered by nutraceuticals products. CHD, Coronary Heart Disease (Das *et al*., 2012)

One of the broader models of organization for nutraceuticals is based upon their potential as a food source to humans. Here nutraceuticals may be classified into plant, animal, and microbial (i.e., bacteria and yeast) groups.

Plants	Animal	Microbial
α-Glucan	Conjugated Linoleic Acid (CLA)	*Saccharomyces boulardii* (yeast)
Ascorbic acid	Eicosapentaenoic acid (EPA)	*Bifidobacterium bifidum*
γ-Tocotrienol	Docosahexenoic acid (DHA)	*B. longum*
Quercetin	Spingolipids	*B. infantis*
Luteolin	Choline	*Lactobacillus acidophilus*(LC1)
Cellulose	Lecithin	*L. acidophilus*(NCFB 1748)
Lutein	Calcium	*Streptococcus salvarius* (subs. Thermophilus)
Gallic acid	Coenzyme Q_{10}	
Perillyl alcohol	Selenium	
Indole-3-carbonol	Zinc	
Pectin	Creatine	
Daidzein	Minerals	
Glutathione		
Potassium		
Allicin		
δ-Limonene		
Genestein		
Lycopene		
Hemicellulose		
Lignin		
Capsaicin		
Geraniol		
β-Ionone		
α-Tocopherol		
β-Carotene		
Nordihydrocapsaicin		
Selenium		
Zeaxanthin		
Minerals		
MUFA		

Figure 3. Examples of nutraceutical substances grouped by food source (Keservani *et al.*, 2010)

Another means of classifying nutraceuticals is by their mechanism of action. This system groups nutraceutical factors together, regardless of food source, based upon their proven or purported physiological properties. Among the classes would be antioxidant, antibacterial, antihypertensive, anti-hypercholesterolemic, antiaggregate, anti-inflammatory, anticarcinogenic, osteoprotective, and so on.

Anticancer	Positive Influence on Blood Lipid Profile	Antioxidant Activity	Anti inflammatory	Osteogenetic or Bone Protective
Capsaicin	α-Glucan	CLA	Linolenic acid	CLA
Genestein	γ-Tocotrienol	Ascorbic acid	EPA	Soy protein
Daidzein	δ-Tocotrienol	β-Carotene	DHA	Genestein
α-Tocotrienol	MUFA	Polyphenolics	GLA (gamma-linolenic acid)	Daidzein
γ-Tocotrienol	Quercetin	Tocopherols		Calcium
CLA	ω-3 PUFAs	Tocotrienols		Casein phosphopeptides
Lactobacillus acidophilus	Resveratrol	Indole-3-carbonol	Capsaicin	FOS(fructooligosaccharides)
Sphingolipids	Tannins	α-Tocopherol	Quercetin	
Limonene	β-Sitosterol	Ellagic acid	Curcumin	Inulin
Diallyl sulfide	Saponins	Lycopene		
Ajoene	Guar	Lutein		
α-Tocopherol	Pectin	Glutathione		
Enterolactone		Hydroxytyrosol		
Glycyrrhizin		Luteolin		
Equol		Oleuropein		
Curcumin		Catechins		
Ellagic acid		Gingerol		
Lutein		Chlorogenic acid		
Carnosol		Tannins		
L. bulgaricus				

Figure 4. Examples of nutraceuticals grouped by mechanisms of action (Keservani *et al.*, 2010)

Botanical materials represent a large segment of nutraceuticals; in this context represent whole, fragmented or cut plants, algae, fungi, lichens and botanical preparations from these materials involving extraction, distillation, fractionation, purification, concentration and fermentation.

Much of the early development of the nutraceutical concept and products was driven from the United States of America where, since its introduction in 1994, the Dietary Supplement and Health Education Act (DSHEA, 1994) has allowed considerable flexibility and blurred the boundaries between foods and medicines found in other parts of the world.

Under DSHEA a dietary supplement may contain ‘an herb or other botanical’ or ‘a concentrate, metabolite, constituent, extract or combination of any ingredient from the other categories’. This is subject to very little qualification and as a consequence a wide variety of botanicals and other substances have been sold as dietary

supplement ingredients, including many that are considered to be medicinal substances under most regulatory regimes in EU countries.

1.1 Botanical nutraceuticals in the European Union (EU)

Within Europe, the regulatory status of nutraceuticals is diversified due to differences in tradition, historical and cultural backgrounds and different legislation and enforcement practices at national level within the 28 member states.

There is currently no consistency in the legal status of some botanicals across the EU. In some EU countries, botanical products are sold as foods, or incorporated in functional/fortified foods or as food supplements, meaning that no medicinal claims are made, whereas in other EU countries these preparations are seen as herbal medicinal products registered by full or simplified registration procedures.

Concepts of "Food Supplements" and "Functional Food"

Food supplements are defined in Article 2 of Directive 2002/46/EC, as "Food stuffs 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 effect, 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, drops dispensing bottles and other similar forms of liquids and powders designated to be taken in measures small unit quantities".

Even after the introduction of this Directive, there is still very little harmonization across the EU, particularly with regard to substances which are neither vitamins nor minerals. Botanical-sourced supplement ingredients are the subject of diverse national legislation; for example, in Italy there is a list of notified herbal food supplement products and another list of herbals prohibited to be incorporated in food supplements. The concept of "positive" and "negative" list is generally accepted within the European countries.

Regarding functional foods, the concept of foods for specified health use (FOSHU) was established in Japan in 1991 (The FOSHU system, 1991): "Foods that are

expected to have certain health benefits, and have been licensed to bear a label claiming that a person using them for a specified health use may expect to obtain the health use through the consumption thereof".
According to Japanese Ministry of Health and Welfare FOSHU are:

• foods that are expected to have a specific health effect due to relevant constituents, or foods from which allergens have been removed;
• foods where the effect of such addition or removal has been scientifically evaluated and permission is granted to make claims regarding their specific beneficial effects on health.

It is in that context that in 1995 the European Commission's concerted action on Functional Food Science in Europe (FUFOSE), actively involving large number of the most prominent European experts in nutrition and related sciences, was engaged by the International Life Science Institute (ILSI) in Europe to propose "a working definition" of functional food (Diplock *et al.*, 1999):
"A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond normal and adequate nutrition, in a way that improves health and well being or reduces the risk of disease. It is not a pill or a capsule, but part of the normal food pattern".

1.2 Polyphenols as potential nutraceuticals

Polyphenols form a large group of phytochemicals, which are produced by plants as secondary metabolites to protect them from photosynthetic stress and reactive oxygen species. In many cases these substances serve as plant defense mechanism against predation by microorganisms, insects and herbivores.
Polyphenols are widely distributed plant-derived dietary constituents and have been implicated as the active components in a number of herbal and traditional medicines (Wollenweber, 1988). More than 5000 plant polyphenols have been identified and

several of them are known to possess a potential wide spectrum of pharmacological properties (Beretz *et al*., 1977).

These metabolites have low potency as bioactive compounds when compared to pharmaceutical drugs, but since they are ingested regularly and in significant amounts as part of the diet, they may have a noticeable long-term physiological effect.

Polyphenols are well known for their antioxidant properties and their abilities to act as scavengers of reactive oxygen species (ROS). ROS are involved in many cellular events, including as second messengers in the activation of several signaling pathways leading to the activation of transcription factors, mitogenesis, gene expression, and the induction of apoptosis, or programmed cell death (Nair *et al*., 2007; Simon *et al*., 2000). Overproduction of ROS, as indicated by a change in the redox state of the cell, may lead to oxidative damage of proteins, lipids and DNA, and it is often an associated risk factor to several common diseases.

Dietary polyphenols have been demonstrated to affect numerous cellular processes like gene expression, apoptosis, platelet aggregation, intercellular signaling, that can have anticarcinogenic and anti-atherogenic implications (Duthie *et al.,* 2003). Polyphenols also possess anti-inflammatory, antimicrobial, cardioprotective activities and play a role in the prevention of neurodegenerative diseases and diabetes mellitus (Scalbert *et al.,* 2005).

Some of the most common polyphenols found in the nutraceutical market belong to the class of anthocyanins, proanthocyanidins, flavonols, stilbenes, hydroxycinnamates, ellagic acid and ellagitannins, isoflavones and lignans, as summarized in Figure 5.

Bioactive compound	Examples	Sources	Putative vasculoprotective effects
Flavonols	Quercetin, kaempferol, catechin	Onion, apple, tea, berries, olives, broccoli, lettuce, red wine, cocoa/chocolate	↓TC, ↓LDL-C oxidation↑HDL-C, AOx, ↓platelet aggregation, ↓eicosanoid synthesis, ↓athero-ELAMs, ↓angiogenesis, ↓MMPs
Flavonols	Epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate	Green/black tea, cocoa/chocolate	AOx, ↓apoptosis, ↓LDL-C oxidation, ↓platelet aggregation, ↓athero-ELAMs, ↓angiogenesis, ↓MMPs
Lignans	Enterolactone, enterodiol	Flaxseed oil, lucerne, clover	↓LDL-C, AOx, estrogen/antiestrogen; ↓atherosclerosis *in vivo* but may show adverse CVD effect (pro-oxidant activity with partially defatted flaxseed)
Isoflavones	Genistein, daidzein	Soybeans, legumes	↓TC and LDL-C, ↓LDL-C oxidation, ↓TG, ↑HDL-C, ↓thrombosis, AOx, estrogen/antiestrogen, ↓athero-ELAMs, ↓angiogenesis, ↓atherosclerosis *in vivo*, ↓MMPs
Stilbenoids	Resveratrol	Grapes, red wine, peanuts	↓LDL-C oxidation, ↓platelet aggregation/thrombosis, ↓eicosanoid synthesis, AOx, ↓athero-ELAMs, ↓angiogenesis but promotes angiogenesis in the ischemic heart, ↓atherosclerosis *in vivo*, ↓MMPs
Carotenoids	Lycopene	Tomatoes, tomato products	↓LDL-C and LDL-C oxidation, Aox, ↓athero-ELAMs, ↓MMPs, but no effects was shown in animal models of ATS and and dietary intervention studies using well-defined subjects population did not provided a clear evidence of lycopene in the prevention of CVD
Carotenoids	α-Carotene β-carotene, γ-carotene, δ-carotene	Carrots, pumpkins, maize, tangerine, orange and yellow fruits and vegetables	Inconsistent data. β-carotene have shown adverse CVD effect because its prooxidant activity
Organosulfur compounds	Allicin, diallyl sulfide, diallyl disulfide, allyl mercaptan	Garlic, onion, leek	↓TC and LDL-C, ↓TG, ↓cholesterol and FA synthesis, ↓BP, ↓thrombosis, AOx, ↓athero-ELAMs, ↓angiogenesis, ↓atherosclerosis *in vivo*, ↓MMPs
Soluble dietary fibers	Glucan, pectin, psyllium	Oats, barley, yeast, fruit, vegetables, psyllium seed, fortified cereals and grains	↓TC, ↓TG, ↓LDL-C
Isothiocyanates	Phenethyl (PEITC), benzyl (BITC), sulforaphanes	Cruciferous vegetables (e.g., watercress, broccoli)	no relevant effects
Monoterpenes	d-Limonene, perillic acid	Essential oils of citrus fruit, cherries, mint, herbs	↓TC and LDL-C, ↓HMGCoAR, ↓angiogenesis
Plant sterols	Sitostanol, stigmasterol, campesterol	Tall oil, soybean oil, rice bran oil	↓TC and LDL-C, AOx, ↓cholesterol absorption; adverse effect: ↓carotenoid absorption
Phenolic acids	Tyrosol, hydroxytyrosol, oleoeuropeine, caffeic acid, cumaric acid	Extra virgin olive oil	↓LDL-C oxidation, ↓platelet aggregation/thrombosis, ↓eicosanoid synthesis, AOx, ↓athero-ELAMs, ↓atherosclerosis *in vivo*, ↓MMPs
ω-3 PUFA	DHA, EPA, αLA	Fish and fish oil, green leaves	↓TC, suppression of cardiac arrhythmias, ↓BP ↓platelet aggregation, ↓eicosanoid synthesis, ↓athero-ELAMs, ↓angiogenesis; ↓MMPs
Prebiotics	Inulin-type fructans	Fruit and vegetable, purified extract from chicory root	↓TC and ↓TG
Probiotics	Selected strains of *Lactobacillus acidophilus, Bifidobacterium bifidum and Lactobacillus bulgaricus*	Fermented milk products	↓TC, LDL-C and BP

AOx, antioxidant activity; BP, blood pressure; CVD, cardiovascular disease; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; MMPs, metalloproteinases; ELAMs, endothelial leukocyte adhesion molecules.

Figure 5. Potential cardiovascular benefits of different nutraceuticals (Massaro *et al.*, 2010)

A large number of phytochemicals-containing nutraceuticals with various compositions and health claims are now widely distributed and available in the market. However, the scientific evidence supporting their health benefits is mostly based on *in vitro* or animal model assays. Clinical trials that evaluate the actual

physiological effects in humans are scarce and results are controversial. There are many factors that may have an impact in the final outcome of the trials, i.e. the stability of the bioactive compounds in the different pharmacological forms available and (or) in the gastrointestinal tract. Any chemical alteration of the original bioactive compound that may take place during storage or digestion may modify severely the bioavailability and bioactivity of the compounds. Another important factor is the inter-individual variability for bioavailability and metabolism as well as for the biological response (Espín *et al*., 2007).

In this regard, it is interesting to remark that several of the health benefits assigned to many dietary constituents are still under controversy as can be deduced from the large number of applications rejected by the European Food Safety Authority (EFSA) about health claims of new foods and ingredients (Gilsenan, 2011). More sounded scientific evidences are needed to demonstrate or not the claimed beneficial effects of these new foods and constituents. In this sense, the advent of new post-genomic strategies as foodomics seems to be essential to understand how the bioactive compounds from diet interact at molecular and cellular level, as well as to provide better scientific evidences on their health benefits.

Many works have been published so far studying the effect of dietary polyphenols on different types of diseases; however, it has been repeatedly indicated that, effects from dietary polyphenols involve multiple molecular and biochemical mechanisms of action, which are still not completely characterized, concluding that many features remain to be elucidated about their claimed activity. Important efforts need to be developed in the next future to implement the knowledge in this intriguing field.

1.3 Foodomics: a new approach to food and nutrition

Currently, there is a general trend in food science to link food and health. Thus, food is considered today not only a source of energy but also an affordable way to prevent future diseases.

Food scientists and nutritionists try to improve our limited understanding of the roles of food compounds at molecular level (i.e., their interaction with genes and their subsequent effect on the expression of proteins and metabolites) for the rational design of strategies to manipulate cell functions through diet, which is expected to have an extraordinary impact on our health.

This trend has given rise to the development of new methodologies in which advanced analytical methodologies, mainly "omics", and bioinformatics – frequently associated to *in vitro*, *in vivo* and/or clinical assays – are applied to investigate topics considered unapproachable few years ago.

Foodomics has been recently defined as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumer's well-being, health, and confidence (Cifuentes *et al*., 2009; Herrero *et al*., 2010; Herrero *et al*., 2012).

Thus, foodomics is presented as a global discipline in which food (including nutrition), advanced analytical techniques (mainly omics tools), and bioinformatics are closely combined. The development of genomics, transcriptomics, proteomics, and metabolomics has given rise to extraordinary opportunities for increasing our understanding about different issues that can now be addressed by foodomics.

It is now well-known that health is heavily influenced by genetics. However, diet, lifestyle, and environment can have a crucial influence on the epigenome, gut microbiome and, by association, the transcriptome, proteome and, ultimately, the metabolome. When the combination of genetics and nutrition/lifestyle/environment is not properly balanced, poor health is a result. Foodomics is a major tool for detecting small changes induced by food ingredient(s) at different expression levels. A representation of an ideal foodomics strategy to investigate the effect of food ingredient(s) on a given system (cell, tissue, organ, or organism) is shown in Figure 6.

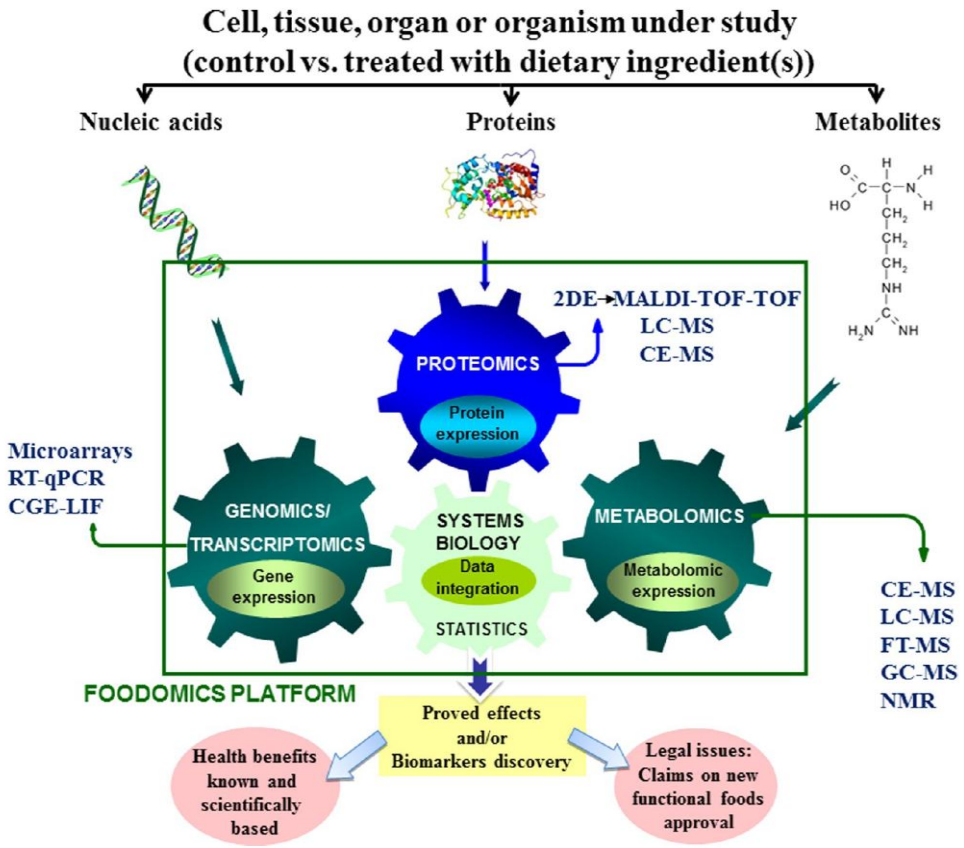


Figure 6. Ideal foodomics platform to investigate the health benefits from dietary constituents on a given biological system (cell, tissue, organ, or organism), including analytical methodologies used and expected outcomes (García-Cañas *et al*., 2012)

Following this foodomics strategy, results on the effect of food or food ingredient(s) at the genomic/transcriptomic/proteomic and/or metabolomic level are obtained, making possible new investigations at the molecular level on food bioactivity and its effect on human health.

Thus, the huge analytical potential of foodomics can allow solving questions related to food safety, traceability, whole quality, transgenic foods, functional foods, nutraceuticals, etc. Foodomics can therefore be an adequate strategy to investigate the complex issues related to prevention of future diseases through food intake. The future effect of suitable diets on human health has been considered a strategic aspect of the European policies as emerge from the new research programs of Horizon 2020.

Figure 7 shows the results from a global foodomics study on the chemopreventive effect of dietary polyphenols against HT29 colon cancer cells (Ibáñez *et al*., 2012), presenting the genes, proteins and metabolites identified (after transcriptomic, proteomic, and metabolomic analysis) that are involved in the principal biological processes altered in HT29 colon cancer cells, after the treatment with rosemary polyphenols. These SFE (supercritical fluid extraction) extracts, rich in dietary polyphenols, were characterized by the presence of cirsimaritin and genkwanine together with terpenoidic compounds.

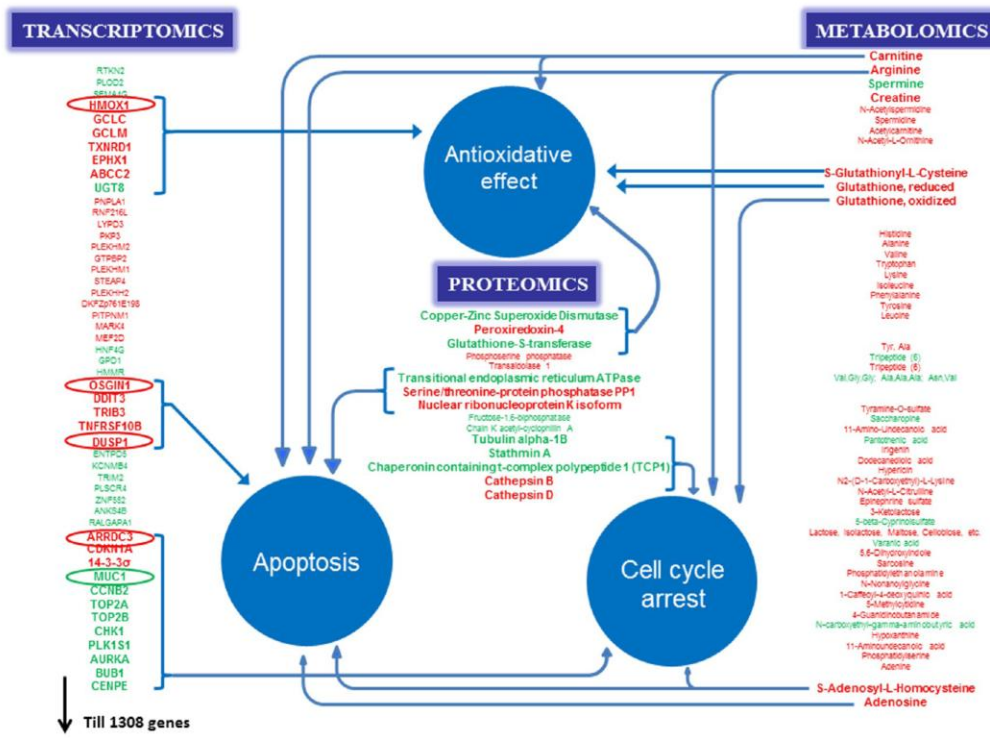


Figure 7. Foodomics identification of the genes, proteins, and metabolites involved in the principal biological processes altered in HT29 colon cancer cells after their treatment with rosemary polyphenols. In red, up-regulated; in green, down-regulated (Ibáñez *et al*., 2012)

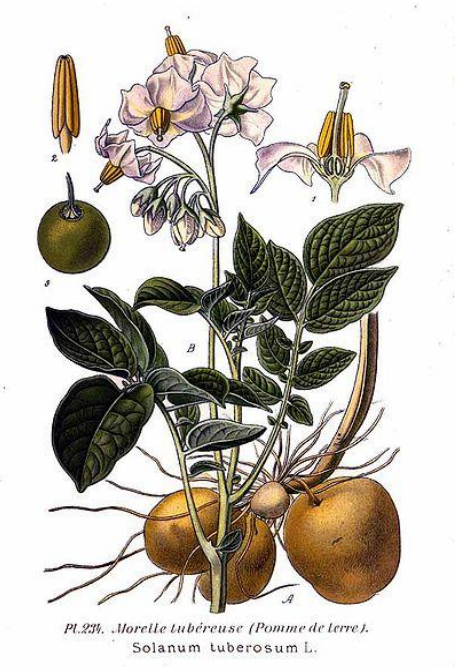
Polyphenols induce various cellular mechanisms that modify the antioxidant activity inside the cell. They bring about an induction of cell-cycle arrest, an increase of apoptosis and an improvement of cellular antioxidant activity (Ibáñez *et*

al., 2012). These antioxidant properties, together with induction of apoptosis and cell cycle arrest can explain the chemopreventive properties of the rosemary polyphenols against colon cancer cells.

The genes, proteins and metabolites identified and implicated in these three processes are shown in Figure 7.

The direct integration following a Systems Biology approach of all the information obtained at the three expression levels is far from being obvious at this moment. The bioinformatic tools capable to handle and integrate all the omics-data generated by different analytical platforms are still distant from what is needed. In summary, this new Foodomics strategy provides an impressive analytical power and it is expected to help to overcome many of the new challenges emerging in Food Science and Nutrition. However, it is observed that together with its remarkable analytical power, new limitations will also come out mainly related to our limited knowledge on the entire cellular mechanisms and the way to handle all the amount of complex multidimensional data and information that can be generated by Foodomics. A rational and holistic combination of all this information will be crucial in order to extract all the biological meaning from the results provided by this new strategy.

2. POTATO: *Solanum Tuberosum* L.



Potato is the fourth most important food crop in the world after rice, wheat and maize, and is the only major food crop that is a tuber.

Potatoes were introduced outside the Andes region four centuries ago, appearing in Europe during the last quarter of the sixteenth century. Traditionally potatoes are a main component of warm meals in many European countries.

Freshly harvested potatoes contain about 80% water and 20% dry matter. About 60–80% of the dry matter is starch. In addition, the potato is low in fat and rich in several micronutrients, especially vitamin C. It is also a good source of vitamins B1, B3, B6, folate, pantothenic acid, riboflavin and minerals, such as potassium, phosphorus and magnesium (FAO, 2008). Apart from being a rich source of starch, potatoes contain good quantity of small molecules and secondary metabolites which play an important role in a number of processes (Friedman, 1997). Many of the compounds present in potato are important because of their beneficial effects on health, therefore are highly desirable in the human diet (Katan & De Roos, 2004).

2.1 Phytochemicals in potato

In addition to supplying energy, potatoes contain a number of health promoting phytonutrients such as phenolics, flavonoids, folates, kukoamines, anthocyanins, and carotenoids.

Polyphenols comprise over 8000 identified substances, which can be divided into groups according to their chemical structure, such as phenolic acids, stilbenes, coumarins, lignins and flavonoids (Ross & Kasum, 2002). Polyphenols are recognized as the most abundant antioxidants in our diet (Manach *et al.*, 2004) and potatoes are a good source of these compounds.

Phenolic compounds represent a large group of minor chemical constituents in potatoes, which play an important role in determining their organoleptic properties (Ezekiel *et al.*, 2013).

Further, phenolics have a wide-array of health providing characteristics (Bravo, 1998), therefore have potential for use as functional food for improving human health.

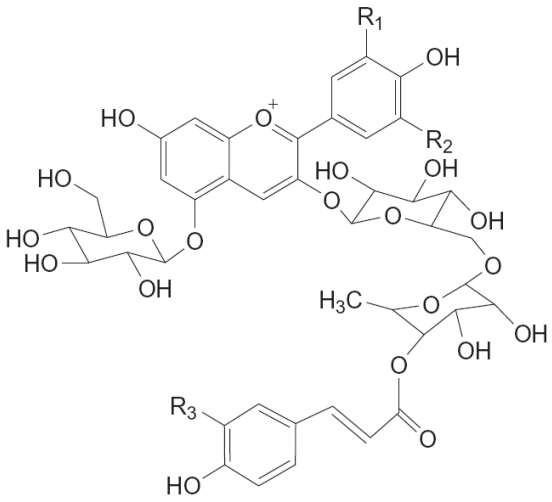
Simple phenols, mainly the analogues of chlorogenic acid, are well known in potatoes and are involved in browning of the tuber after cutting or processing (Dao & Friedman, 1992). For the most part, these compounds are localised in skin (nearly 50% of the total content) and the percentage decreases gradually going inwards in the tuber (Friedman, 1997). The fresh pulp contains from 30 to 900 mg/kg of chlorogenic acid and minor amounts of other phenolic acids (0–30 mg/kg), but in the skin up to 1000–4000 mg/kg of chlorogenic acid can be present (Lewis *et al.*, 1999).

Purple- and red-skinned tubers contained twice the concentration of phenolic acids as white-skinned tubers. It was also reported that purple- or red-fleshed cultivars had three to four times the concentration of phenolic acids of white fleshed cultivars. The phenols can be recovered from the skin portion, which is discarded as waste during potato processing and can be used for ‘value addition’ in different food products (Navarre *et al.*, 2009).

Flavonoids content in potatoes ranged from 200 to 300 μg/g FW (Lewis *et al.*, 1998). The flavonoids, in order of abundance, were reported to be catechin, epicatechin, erodictyol, kaempeferol and naringenin (Brown, 2005). Anthocyanins are natural pigments belonging to the flavonoid family, present in substantial amounts in pigmented flesh potatoes. Until now fresh fruits and red wine have been commonly indicated as rich sources of anthocyanins in the human diet, but also red-blue potatoes can contribute to increasing the intake of these interesting pigments. Anthocyanin levels between 5.5 and 35 mg/100 g FW in potatoes have been reported (Brown, 2008). Lewis *et al.* (1998) found that purple or red-fleshed cultivars had twice the flavonoid concentration of white-fleshed cultivars and their concentrations are considerably higher in skin, approaching 900 mg in purple-fleshed and 500 mg in red-fleshed types per 100 g FW. Anthocyanin pigments in the periderm of potatoes impart different colours to their skin, purple being the most common colour.

The pigmented potatoes may serve as a potential source of natural anthocyanin pigments, being low-cost crops (Jansen & Flamme, 2006), and also a powerful source of antioxidant micronutrients (Andre *et al.*, 2007). The purple- and red-fleshed potatoes could be used as novel sources of natural colourants and antioxidants by the food industry for better human health (Reyes *et al.*, 2007). Anthocyanidins (the aglycon form of anthocyanins) are present in fruits and vegetables linked to one or more glycosidic units. Sugars may be linked as mono, di or triglycosides and may, in addition, be acylated with different organic acids.

To date, 23 basic anthocyanidins have been identified; red- and purple fleshed potatoes had acylated glucosides of pelargonidin, while purple potatoes had, in addition, acylated glucosides of malvidin, petunidin, peonidin, and delphinidin (Brown, 2005; Lachman & Hamouz, 2005).



	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delfinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

	R_3
Ferulic acid	OCH_3
Caffeic acid	OH
P-cumaric acid	H

Figure 8. General chemical structure of the anthocyanins found up date in *Solanum tuberosum* L. (Ieri *et al.* 2011)

Potatoes also contain folates, though in limited concentration; they are a major source of these compounds due to their higher consumption. Potatoes supply about 10% of the total folate intake of the people in European countries such as Netherlands, Norway and Finland (Navarre *et al.*, 2009). Folate concentrations in potato vary between 12 and 37 µg/100 g FW (Konings *et al.*, 2001). Folate content in more than 70 potato cultivars, advanced hybrids and wild species has been reported to range from 11 to 35 µg/100 g FW (Goyer & Navarre, 2007). Higher folate content was generally reported to be present in yellow fleshed potatoes (Ezekiel *et al*, 2013).

Kukoamines are polyamine conjugates and considered to have health promoting effects, which are yet to be well established. Kukoamines in potatoes was first reported by Parr *et al.* (2005). Tuber polyamines have been suggested to play a role in the regulation of starch biosynthesis (Tanemura & Yoshino, 2006) and making the tubers resistant to diseases (Matsuda *et al.*, 2005).

Potatoes are a good source of carotenoids, lipophilic compounds synthesized in plastids from isoprenoids (Dellapenna & Pogson, 2006). Lutein, zeaxanthin, violaxanthin and neoxanthin are the major carotenoids present in potatoes and β-carotene is present in trace amounts. The orange and yellow colour of the tuber flesh is due to zeaxanthin and lutein, respectively. Potato cultivars with white flesh contained less carotenoids as compared to cultivars with yellow or orange flesh. Total carotenoids content was reported in the range of 50–350 μg/100 g FW and 800–2000 μg/100 g FW, respectively, in white- and yellow-fleshed potato cultivars (Brown, 2008).

As part of the potato plant's natural defences against fungi and insects, its leaves, stems and sprouts contain high levels of glycoalkaloids (usually solanine and chaconine), toxic compounds that are normally found at low levels in the tuber, and occur in the greatest concentrations in the periderm. The concentration of glycoalkaloids is related to the genotype and cultivar, as well as environmental factors during growth, harvest and storage. It has been proven that cooking procedures have only small effects on these compounds (Mulinacci *et al.*, 2008). Therefore to guarantee food safety, determination of their content in tubers is required: a maximum level of 200 mg/kg per whole fresh tuber has been established for their commercialisation (Clayton & Percival, 2000).

2.2 Factors affecting phytochemicals content and stability

2.2.1 Genotype

The number of potato varieties known to mankind is vast, estimated to be approximately 5000. About 11 *Solanum* species are cultivated but most of the potato varieties cultivated throughout the world belong to the species *Solanum tuberosum*. Apart from these, about 200 wild species are known to exist. The nutrient content of potatoes was reported to be influenced by a number of factors, variety being the most important (Toledo & Burlingame, 2006). A large variation exists in the phytonutrients content of the several cultivars of potato.

Using hundreds of potato genotypes, Navarre *et al.* (2009) found up to fifteen-fold difference in their phenolic content. White-fleshed potato varieties were reported to contain lower amount of phenolics (less than 4 mg/g DW) as compared to purple-fleshed wild species (more than 5–6 mg/g DW). An anthocyanin content of up to 7 mg/g FW in the skin and 2 mg/g FW in the flesh was reported by Lewis *et al.* (1998) amongst 26 potato cultivars with coloured flesh. Pelargonidin and peonidin were reported to be present in nearly equal amounts in the red flesh, while petunidin and malvidin were predominant in the purple flesh.

Jansen & Flamme (2006) analysed 31 potato genotypes with coloured flesh and found a lower range of 0.5 to 3 mg/g FW in the skin and up to 1 mg/g FW in the flesh, while Brown *et al*. (2005) determined the anthocyanin content in several genotypes and reported a value of up to 4 mg/g FW in whole tubers.

Eichhorn & Winterhalter (2005) identified major anthocyanins present in four pigmented potato cultivars. Petunidin derivatives were detected in three varieties, pelargonidin was found to be the only anthocyanidin in cv. "Highland Burgundy red", malvidin was the predominant aglycon of the cv. "Vitolette"' and minor amounts of peonidin derivatives were found in cv. "Shetland Black". Jansen and Flamme (2006) analyzed 27 potato cultivars and observed that the average anthocyanins content was the highest in the skin (0.65 g/kg FW). The corresponding values for whole tubers and flesh were 0.31 g/kg FW and 0.22 g/kg FW, respectively; the average anthocyanin content was higher in violet coloured potatoes and lower in red coloured potatoes.

2.2.2 Agronomic Factors

Phytonutrients content of potatoes may be influenced by developmental stage. Potatoes harvested at a young developmental stage had higher concentrations of some phytonutrients such as folate and chlorogenic acid than mature tubers (Goyer & Navarre, 2007; Navarre *et al.*, 2010). Total carotenoids content was found to be higher in immature tubers and it decreased with tuber maturity (Kotikova *et al.*, 2007; Morris *et al.*, 2004). Reyes *et al.* (2004) observed that the anthocyanins and total phenolic content in tubers decreased with tuber growth and maturity but total

yield per ha of these compounds increased through time. Harvesting at later maturity stages maximized total yield of potatoes, anthocyanin and total phenolic content, and minimized glycoalkaloid content, thus increasing the commercial and nutritional value of purple and red-flesh potatoes.

Effect of location of crop growth (coastal area and plains) on phytochemicals content has been studied by several researchers and does not seem to have a significant effect on the anthocyanin content of tubers (Jansen & Flamme, 2006). According to Kotikova *et al.* (2007), the level of anthocyanins was not affected by the environmental conditions and it was primarily dependent on the genotype. Potatoes grown at two locations differing in altitude showed no significant difference in the total carotenoids content.

However, other studies showed different results: a significant effect of location on anthocyanin and total phenolics was observed by Reyes *et al.* (2004). The anthocyanins and total phenolic content of potato tubers was enhanced when tubers were grown in a location with cooler temperatures and longer days (higher solar radiation) with up to 2.5 and 1.4 times, respectively, higher anthocyanins and total phenolic content found under such conditions. Hence it appears that temperature during crop growth can affect the phytochemicals content. In the study carried out by Brown *et al.* (2008), potatoes were grown at 3 locations varying in altitude (203, 960 and 1250 masl); higher anthocyanins content were observed at higher elevations, however total carotenoids were not affected.

Conflicting results have also been reported with respect to year of crop growth. Jansen and Flamme (2006) compared the anthocyanin contents in tubers of 23 cultivars grown during two years and found that there was no significant difference between years in the anthocyanin content of tubers, although the weather conditions during plant growth were different during the two years. Contrarily Stushnoff *et al.* (2008) and also Rosenthal & Jansky (2008) observed environmental conditions produced year to year variation in total phenolics levels.

2.2.3 Postharvest Storage

Storage generally increases total phenols content in potatoes but little change or a decrease in phenols content after storage have also been reported in some studies. Ezekiel & Singh (2007) determined total phenols content in four potato cultivars stored for 180 days at 4, 8, 12, 16 and 20 °C; total phenols increased after storage and the increase was higher at 4 and 16 °C. In another study Ezekiel *et al.* (2000) found that the total phenols in potato tubers continued to increase up to 271 days of storage at 6 °C but at 20 °C, it decreased after 220 days of storage.

Effect of storage of potatoes at 4 or 20 °C for 110 days on phenolic content was studied by Blessington *et al.* (2010). No significant differences in total phenolic content, chlorogenic acid, caffeic acid and vanillic acid were observed after storage at 4 or 20 °C. There was an increase in rutin, p-coumaric acid and quercetin dehydrate contents after storage at 4 or 20 °C. When 4 °C stored potatoes were reconditioned for 10 days at 20 °C, there was a significant increase in total phenolic content, chlorogenic acid, caffeic acid, rutin, vanillic acid, p-coumaric acid, and quercetin dehydrate levels. All the three storage treatments resulted in increased carotenoid content but caused no significant differences in phenolic content and antioxidant activity in most of the eight genotypes studied. Stushnoff *et al*. (2008) analysed total phenolics from 8 potato genotypes after 112 and 263 days of storage at 5 °C. Two genotypes showed sharp rise in total phenolics after storage, four genotypes showed increase to a lesser extent and two genotypes showed little change.

Rosenthal & Jansky (2008) observed that stored tubers had higher levels of antioxidant activity than fresh tubers. Jansen & Flamme (2006) determined the anthocyanin content of tubers in 14 cultivars immediately after harvest and after 135 days of storage at 4 °C and 86% relative humidity, and did not find any significant change in anthocyanin content of tubers. The fact that cold storage had no significant effect on the anthocyanin content of potatoes indicates that there is no risk of degradation of these compounds during storage of potatoes over a longer period.

2.2.4 Cooking and Processing

Attempts to increase phytonutrients content in potatoes will become futile if the targeted phytonutrients do not survive cooking in reasonable quantities (Navarre *et al.*, 2010).

Potato peels have been shown to contain a high quantity of phenolics; boiling and baking potatoes with skin was considered to be a good method of cooking as it helped in retaining most of the nutrients.

Mondy & Gosselin (1989) found that the potatoes cooked with peel had a greater amount of total phenols in the cortex and internal tissues. This has been attributed to the migration of phenolics from the peel into both the cortex and internal tissues of the tuber. Barba *et al.* (2008) observed significant losses in phenolic contents between peeled and unpeeled potatoes, and between boiling and baking. The losses were observed to be lower in unpeeled potatoes. For example, the losses in caffeoylquinic acids were 20.6 and 26.8%, respectively in unpeeled and peeled potatoes after microwave baking against losses of 24.1 and 25.7%, respectively, after conventional boiling. The change in phenolic content during processing was attributed to the combination of losses caused by leaching into water, degradation from the effects of heat, oxidation by polyphenol oxidase, and isomerisation (Takenaka *et al.* 2006). Also Faller & Fialho (2009) reported that boiling, microwave baking and steaming decreased the polyphenols content. However, the recovery of polyphenols was higher after boiling as compared to microwave cooking and it was least in steamed potatoes. They observed that unlike in other vegetables such as carrot, onion and cabbage, cooking caused an increase in antioxidant capacity in potatoes, nevertheless various cooking methods did not show significant differences. The formation of novel substances, such as products of Maillard reaction, could also increase the antioxidant capacity in potatoes (Manzocco *et al.*, 2000). Dao & Friedman (1992) observed complete destruction of chlorogenic acid during baking and 60% reduction by microwaving, while Im *et al.* (2008) observed less than 5% losses in chlorogenic acid during baking of potatoes. Navarre *et al.* (2010) determined losses in phenolics after cooking by microwaving, steaming, boiling or baking and found that none of these cooking methods

decreased the amount of chlorogenic, cryptochlorogenic and neochlorogenic acids. According to Mulinacci *et al.* (2008), boiling and microwaving did not cause any changes in the phenolic acids content but caused 16–29% decrease in anthocyanins content.

Basing on the study of Blessington *et al.* (2010), carotenoid content was observed to be lower in boiled as compared to raw potatoes, however, no significant difference in other methods of cooking was observed. The total phenolic content and antioxidant activity did not show any difference between raw and boiled potatoes but were higher in baked, fried or microwaved potatoes. Greater amounts of phenolics may be extracted out of the potato matrix into water during boiling or into the oil during frying. It appears that anthocyanins and carotenoids withstand the usual modes of cooking and retain their antioxidant capacity after cooking (Brown, 2005).

Loss of nutrients during processing is a major concern and it is desiderable to minimize nutrient losses during conversion of raw potatoes into various products. Minimal processing such as handling, washing and cutting can cause changes in phytochemicals and can lead to activation of some enzymes which modify the level of phenolic compounds (Tudela *et al.*, 2002).

Wounding of fresh potatoes has been shown to cause changes in phenolic compounds and antioxidant capacity (Reyes *et al.*, 2007). Wounding response was cultivar dependent and was reported to increase the phenolic content and antioxidant capacity of purple-flesh potatoes (Reyes & Cisneros-Zevallos, 2003), but decreased total soluble phenolics and antioxidant capacity to the extent of 15% and 51%, respectively, in white-flesh potatoes (Reyes *et al.*, 2007).

Anthocyanins stability during processing was influenced by several factors such as temperature, pH, presence of enzymes, proteins and metallic ions. Thermal processing was reported to cause anthocyanin degradation. Anthocyanins were enzymatically degraded in the presence of polyphenol oxidase, which can be inactivated by mild heating or blanching. Anthocyanins and other phenolic compounds was easily oxidized and, thus, susceptible to oxidative degradation during various steps of processing (Patras *et al.*, 2010).

2.3 Antioxidant activity and health benefits

Potatoes have several secondary constituents with antioxidant activity, which contributes to the physiological defence against oxidative and free-radical-mediated reactions. Potatoes contain water-soluble antioxidants that act as free radical acceptors, e.g. glutathione, ascorbic acid, quercetin and chlorogenic acid. The levels of antioxidants were reported to vary with the flesh colour of potatoes and greater antioxidant activity was observed in skin tissue as compared to flesh. Water soluble anthocyanins are potent antioxidants, but antioxidant activity is not associated only with coloured flesh of potatoes; the colourless compounds, probably either flavonoids or phenolic acids, are potentially potent antioxidants (Brown, 2005).

Potato peel is a good source of natural antioxidants, which has been studied in various food systems (Rodriguez de Sotillo *et al.*, 1994). Potato peel extract provides protection against acute liver injury (Singh *et al.*, 2008) and oxidative damage to erythrocytes (Singh & Rajini, 2008). Thompson *et al.* (2009) have reported that the phytochemicals of freeze-dried potato powder caused a 23% reduction in induced breast cancer in rats. Several other health promoting effects (longevity, heart and eye health) and therapeutic properties (antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, antithrombotic, and vasodilatory activity) of phenolics has been reported (Alan & Miller, 1996; Manach *et al.*, 2004). Many of these effects result from powerful antioxidant and free radical scavenging properties of phenolic compounds (Amakura *et al.*, 2000).

Chlorogenic acid has a strong antioxidant activity and potatoes are an excellent source. This compound is well known for health promoting effects such as protection against degenerative diseases, cancer, heart disease (Nogueira & do Lago, 2007), hypertension (Yamaguchi *et al.*, 2007) and viral and bacterial diseases. Chlorogenic acid has been found to be a strong and selective inhibitor of matrix metalloproteinase (MMP)-9, an angiogenic enzyme responsible for tumor invasion and metastasis (Jin *et al.*, 2005). Chlorogenic acid also slows down the release of glucose into the blood-stream (Bassoli *et al.*, 2008), hence could be helpful in lowering the glycaemic index of potatoes. Therefore, potatoes with lower glycaemic

index are good for diabetic patients and may even decrease the risk of type II diabetes (Legrand & Scheen, 2007). It has been reported that chlorogenic acid blocks nitrosamine formation through competitive reaction with nitrite and to bind the carcinogen benzo(a) pyrene in a cellulose model system (Friedman, 1997). Chlorogenic acid has been demonstrated to exhibit several desirable anticarcinogenic properties including inhibition of A549 human lung cancer cells (Feng *et al.*, 2005).

In the past decade, great interest has developed also regarding evaluation of the anthocyanin content in the human diet and it has been demonstrated that these pigments are rapidly adsorbed at the stomach level (Passamonti *et al.*, 2003) and were detectable in urine and plasma (Harada *et al.*, 2004), where they protect LDL against oxidation (Kano *et al.*, 2005). It is well established that anthocyanins inhibit digestive enzyme activity, such as R-glucosidase, and they can reduce blood glucose levels after starch-rich meals (McDougall & Stewart, 2005; McDougall *et al.*, 2005).

Zhang *et al.* (2005) reported that anthocyanidins delphinidin, pelargonidin, petunidin and malvidin inhibited MCF-7 breast cancer cell cultures.

Anthocyanins are known to prevent cardiovascular diseases, cancer and diabetes (Konczak & Zhang, 2004; Reddivari *et al.*, 2007). Cyanidin has been found to be three times more effective than pelargonidin as an antioxidant (Pietta, 2000), while another study found malvidin as the most potent antioxidant of the anthocyanidins (Kahkonen & Heionan, 2003).

Coloured potato extracts have been reported to suppress lymph-node carcinoma of the prostate and prostate cancer cell proliferation (Reddivari *et al.*, 2010). Polyphenol and anthocyanin rich purple potato flakes were found to play an important role in the protection against adverse effects related to oxidative damage in rats fed a high-cholesterol diet and red potato flakes improved the antioxidant system by enhancing hepatic superoxide dismutase mRNA in rats (Han *et al.*, 2007). In a recent study (Kaspar *et al.* 2010) have been assessed the effects of consumption of pigmented potatoes on oxidative stress and inflammation biomarkers in healthy adult males and was hypothesized that carotenoids and

anthocyanins from pigmented potatoes would decrease oxidative stress and inflammation in humans. In the study of Vinson *et al.* (2012) was also reported that purple potatoes are an effective hypotensive agent and lower the risk of heart disease and stroke in hypertensive subjects without weight gain.

It has been reported that also flavonoids have shown antioxidant activity and differ significantly in their antioxidant capacity (Pietta, 2000). Quercetin was found to be three times more effective as an antioxidant than kaempferol and eridictyol, and was thrice as effective as catechin. Reyes *et al.* (2005) observed a high positive correlation between antioxidant capacity and anthocyanin and phenolic content, concluding that these compounds are mainly responsible for the antioxidant capacity.

Potatoes contain enough phytochemicals to justify the claim of being health promoters, therefore their use as a substantial part of our daily diet may be recommended. The processing of pigmented potato based foods needs to be considered especially with respect to the antioxidant capacity and other health benefits.

3. ROSEMARY: *Rosmarinus Officinalis* L.

Mediterranean basin is endowed with a rich wealth of aromatic plants as rosemary, *Rosmarinus officinalis* L. (Labiatae), a small evergreen shrub whose health benefits of its essential oil as well as for preservation of food are recognized since ancient times.

Nowadays rosemary is widely cultivated all over the world as ornamental and aromatic plant and the interest towards its cultivation is due to the many biological activities of the essential oil and the extracts obtained from the flowering aerial tops: leaves, twigs and flowers.

Rosemary is known for its numerous applications in the food field and also for the increasing interest in its pharmaceutical properties. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction and the phenolic constituents. Phenolics are mainly represented by rosmarinic acid, by a flavonoidic fraction and by some diterpenoid compounds structurally derived from the carnosic acid.

The volatile compounds, which constitute the essential oil, can be commonly obtained by steam distillation method which gives high yields of a product of appreciable quality on the basis of sensory evaluation, with remarkable functional properties.

3.1 Bioactive phenolic compounds from rosemary

Nowadays rosemary is one of the most appreciated sources for natural bioactive compounds which are of special interest in the functional food industry. In fact this plant exerts a great number of pharmacological activities and most of these

observed effects are linked to the phenolic content of this herb. Rosemary shows potent antioxidant activity (Pérez-Fons *et al.*, 2010; Botsoglou *et al*., 2009; Hernández- Hernández *et al*., 2009), mainly due to phenolic diterpenes, such as carnosol, carnosic acid and rosmanol, among others. Nevertheless, the presence of other antioxidant phenolic compounds in rosemary has also been reported, such as flavonoids (genkwanin, cirsimaritin), and phenolic acids (rosmarinic acid) (Cuvelier *et al.*, 1996; Almela *et al*., 2006).

In the last few years, there has been a growing interest in the use of natural antioxidants, not only for their usefulness as a preservation method but also because of their benefits in human health. These natural antioxidants can protect the human body from free radicals and could retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods, without changing the sensory qualities of the food products (Arts *et al.*,2005; Williamson *et al.,* 2005; Ibanez *et al.*, 2000). Among natural antioxidants of herbal origin, rosemary is one of the most used and commercialized, because of its high content in phenolic compounds.

More than 50 different compounds were identified in rosemary extract (Mulinacci *et al.*, 2011; Borrás Linares *et al.,* 2011), among them the rosmarinic acid, numerous minor flavonoids, either as aglycones and glycosides, and the diterpenoidic constituents. Some of them are reported in Table 1. The rosmarinic acid is generally the main component of the phenolic fraction.

	Compounds	Rts (min)	mw (aglicone)	MS (-)	MS(+)
1	Caffeic acid	7.7	180	135, 179,	181
2	Flavonoid monoglicoside	13.4	478 (316)	315, 477,	317, 479
3	Apigenin rha-glu	15.5	578 (270)		
4	Esperidin rha-glu	15.8	610 (302)	301, 609	
5	Diosmin rha-glu	17.4	608 (300)	299, 607	
6	Luteolin 7-O gluc	17.9	462 (286)	285, 461	
7	Ispidulin 7-O glu	18.5	462 (300)	461	
8	Rosmarinic acid	18.8	360	161, 197, 359, 719	
9	Flavonoid diglicoside	20.8	640 (316)	639	
10	Cirsimaritin O-glu	22.0	476 (316)	315, 475	
11	Flavonoid diglicoside	23.0	654 (316)	653	
12	Isoscutellarein 7-O-glu	23.1	462 (286)	285, 461	
13-18	Flavonoids	24 - 31			
19-20	Rosmanol/epirosmanol	34.4 -35.1	346	283, 345	
21	Cirsimaritin	35.2	314	313	
22	Flavonoid	35.7			315
23	Genkwanine	38.4	284	283	285
24	Flavonoid	41.2	-	-	329, 351
25	Carnosol	41.6	330	285, 329	331, 353
26	4'Metoxytectochrysin	42.2	298		299
27	Carnosic acid derivative	42.4	374		375, 397
28	Carnosic acid	42.8	332	287, 331	
29	Methyl carnosate	43.3	346	287, 331	

Table 1. Main phenolics compounds detected in rosemary extracts (Mulinacci *et al.*, 2011). *Glu, glucose; gluc, glucuronic acid; rha, rhamnose*

It is well known that carnosol and carnosic acid are the strongest antioxidant compounds; it has been reported that approximately 5% of the dry weight of rosemary leaves contains carnosol and carnosic acid and this fraction is estimated to account for >90% of the antioxidant activity (Aruoma *et al*., 1992).

The instability of this phenolic diterpene in the presence of oxygen has been demonstrated. This instability gives rise to new compounds resulting from the breakdown of carnosic acid, such as carnosol, rosmanol, epirosmanol, epiisorosmanol, rosmadial and methylcarnosate (Doolaege *et al.*, 2007).

In the investigation of Zhang *et al.* (2012), degradation of carnosic acid, carnosol and rosmarinic acid in ethanol solution were studied and a new oxidative pathway of carnosic acid was proposed (Figure 9). Carnosic acid quinone was likely to be the

intermediate in the pathway. It was confirmed that rosmanol, epirosmanol, and epirosmanol ethyl ether were generated from carnosol which is a degradation product of carnosic acid. Also, 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone and the light induced degradation product, compound 11, were reported as degradation products of carnosic acid for the first time.

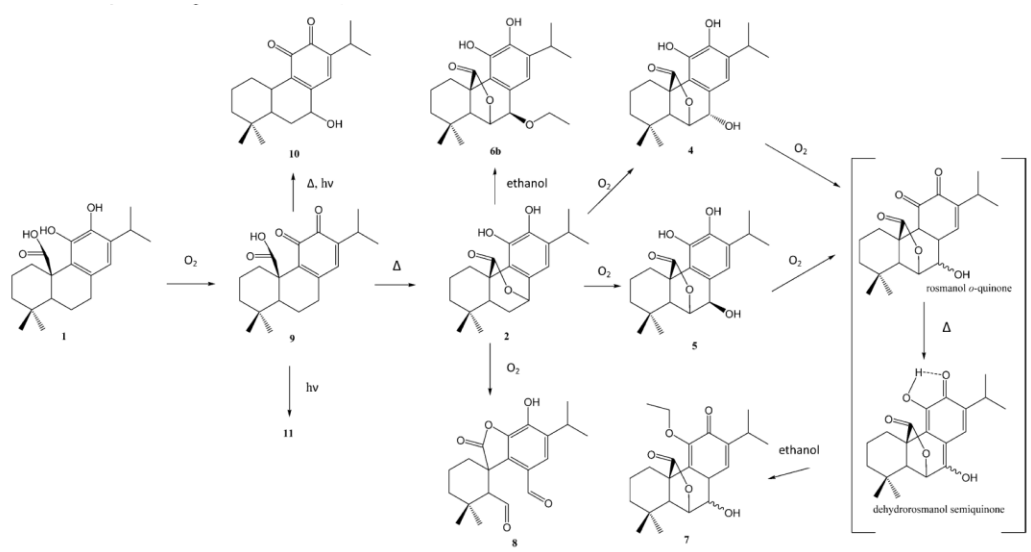


Figure 9. Proposed degradation pathway of carnosic acid (1) in ethanol solution. 2, carnosol; 4, rosmanol; 5, epirosmanol; 6b, epirosmanol ethyl ether; 7, 11-ethoxyrosmanol semiquinone; 8, rosmadial; 10, 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone; 11, light induced degradation product of carnosic acid (structure unknown) (Zhang *et al.,* 2012).

Besides acting as antioxidants, rosemary extract and its constituents have also displayed useful physiological and medicinal properties.

A synergistic antioxidant effect between an extract from rosemary leaves and butylated hydroxytoluene (BHT) and a synergistic interaction with butylated hydroxyanisole (BHA) to inhibit *Escherichia coli* and *Staphylococcus aureus* growth were demonstrated. Therefore, rosemary not only enhances the antioxidant efficiency of BHA and BHT, but also the antibacterial effect of BHA, allowing a decrease from 4.4- to 17-fold in the amounts of the synthetic compounds used (Romano *et al.,* 2009). Moreover the *in vitro* antimicrobial effect of rosemary extract against several bacteria was already showed by Del Campo *et al.* (2000) and

Mahady *et al.* (2005). Rosemary extract was also reported to show inhibitory effects for human immunodeficiency virus (HIV) infection at very low concentrations (Aruoma *et al.,* 1996).

According to the ESCOP (European Society Cooperative on Phytotherapy), ethanol and aqueous extracts from rosemary leaves are used as coleretic, colagogue and hepatoprotective agents, but also as light diuretic, antiulcer, antitumor and antiviral products. Sotelo-Felix *et al.* (2002) reported that rosemary may alleviate carbon tetrachloride-induced acute hepatotoxicity in rats, possibly blocking the formation of free radicals generated during CCl(4) metabolism and hypothesized that this protective effect is due to carnosol.

Recently, by *in vitro* test on hepatic stellate cells, rosmarinic acid showed antifibrogenic effects (Li *et al.,*2009).

Anti-angiogenic potential of rosmarinic acid relating to its antioxidant properties (Shuang-Sheng *et al.,* 2006) and its ability to suppress retinal neovascularization in a mouse model of retinopathy were recently pointed out (Kim *et al.,* 2009). These latter findings suggest this molecule could be used in the treatment of vasoproliferative retinopathies.

Some of the health promoting properties of rosemary have been attributed to the antioxidant activity of polyphenols present in these extracts. Reactive oxygen species (ROS) and depletion of anti-oxidant enzymes have been suggested to promote a variety of biological responses including neurodegenerative, inflammatory conditions, cardiovascular disease, and carcinogenesis of various tissues.

One study (Fortes *et al.,* 2003) observed an inverse relationship between consumption of Mediterranean herbs such as rosemary with lung cancer, suggesting that the phytochemicals isolated from this plant should be investigated for their medicinal properties.

Considerable evidence demonstrates that rosemary extracts, or its isolated components, can inhibit both the initiation and tumor promotion stages of carcinogenesis in mice and rat models.

A methanol extract of the leaves of *Rosmarinus officinalis* L. was evaluated for its effects on tumor initiation and promotion in mouse skin (Huang *et al*., 1994); the application of rosemary inhibited the covalent binding of benzo(*a*)pyrene [B(*a*)P] to epidermal DNA inhibiting tumor initiation by B(*a*)P and 7,12-dimethylbenz[*a*]anthracene (DMBA). Application of rosemary to mouse skin also inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase activity, TPA-induced inflammation, arachidonic acid-induced inflammation, TPA-induced hyperplasia and TPA-induced tumor promotion. According to this study, topical application of carnosol or ursolic acid isolated from rosemary inhibited TPA-induced ear inflammation, ornithine decarboxylase activity and tumor promotion.

Carnosol has been reported to have broad anticancer properties in several cell line models including prostate, breast, leukemia as well as others (Johnson, 2011). The anti-cancer properties of carnosol were associated with a potential to modulate multiple signaling pathways such as the cell cycle related proteins, PI3K/AKT, and apoptotic related proteins (Khan *et al*., 2007).

Offord and co-workers (1995) demonstrated that rosemary extract inhibited the genotoxic effects of the lung procarcinogen B(*a*)P in human bronchial epithelial cells, BEAS-2B, and that strong antioxidant components, carnosol and carnosic acid, were responsible for this effect. The study carried out by Singletary *et al*. (1996) showed that carnosol can prevent DMBA-induced DNA damage and tumor formation in the rat mammary gland and thus, has potential to be used as a breast cancer chemopreventive agent. This role of carnosol in preventing DMBA-induced mammary tumorigenesis may be partially explained by carnosol inducing detoxification enzymes including glutathione-S-transferase (GST) and quinone reductase which carnosol has been shown to modulate in other studies.

Offord *et al*. (1997) studied also the chemoprotective effects of rosemary extract in human liver and bronchial cells and pointed out two mechanisms; one of which was inhibition of the metabolic activation pathway catalyzed by the phase I cytochrome P450 enzymes and the other was induction of the detoxification pathway catalyzed by the phase II enzymes, such as glutathione S-transferase. In another in-depth

study by Huang and co-workers (2005), carnosol is shown to inhibit the invasion of highly metastatic mouse melanoma B16/F10 cells *in vitro*.

The anticarcinogenic activity of soxhlet and supercritical CO_2 rosemary extracts, as well as their active compounds carnosic acid and rosmarinic acid, have been studied versus various human cancer cell lines including NCI-H82 (human, small cell lung, carcinoma), DU-145 (human, prostate, carcinoma), Hep-3B (human, black, liver, carcinoma, hepatocellular), K-562 (human chronic myeloid leukemia), MCF-7 (human, breast, adenocarcinoma), PC-3 (human, prostate, adenocarcinoma), and MDA-MB-231 (human, breast, adenocarcinoma) (Yesil-Celiktas *et al.,* 2010). Carnosic acid, alone or in combination with the anticancer drugs, may offer a good strategy for the treatment of a variety of human cancers that are resistant to chemotherapy.

Rosemary leaf extracts and carnosic acid have been shown to reduce body weight, fat mass gain and serum lipids levels in male mice fed a high-fat diet (Harach *et al.*, 2010; Ibarra *et al.,* 2011) and in a leptin-deficient (ob/ob) male mouse model (Wang *et al.,* 2011). Administration of a dose of rosemary leaf extract (200 mg/kg body weight) was effective to limit weight gain induced by a high-fat diet and protected against obesity-related liver steatosis in mice (Harach *et al.*, 2010). Supplementing the diet with supercritical fluid rosemary extract, containing 20% carnosic acid, reduced oxidative stress in aged rats (Posadas *et al.,* 2009). Long-term dietary administration of ground rosemary at a 1% (w/w) level in the diet improved the antioxidant status of rat tissues following carbon tetrachloride intoxication (Botsoglou *et al.,* 2010). The study of Romo Vaquero *et al.* 2012 showed a significant inhibition of gastric lipase in the stomach of Zucker rats consuming rosemary extract enriched in carnosic acid which may cause a moderate reduction of fat absorption consistent with the observed reduction in weight gain and triglycerides and cholesterol levels. These data suggest that long-term consumption of rosemary extracts rich in carnosic acid may be beneficial for maintaining a normal lipid profile and a lower weight.

The study carried out by Bakirel *et al.* (2008) revealed a defined role of the ethanolic extract of rosemary in suppressing blood glucose level in

normoglycaemic, glucose-hyperglycaemic and alloxan-induced diabetic rabbits, possibly due to its multiple effects involving both pancreatic and extra-pancreatic mechanism. It has also determined that the extract possessed a capability to inhibit the lipid peroxidation and activate the antioxidant enzymes (SOD and CAT) in diabetes.

An use of *Rosmarinus officinalis* in the treatment of depression was reported (Heinrich *et al.*, 2006). Machado *et al.* (2009) have sought to investigate the effect of the hydroalcoholic extract of rosemary in forced swimming test (FST) and tail suspension test (TST), predictive models of antidepressant activity, finally reporting that *Rosmarinus officinalis* produces a specific antidepressant-like effect in both FST and TST. Moreover, the effect of the acute or repeated administration of this extract was similar to the action produced by the classical antidepressant fluoxetine. In addition, it was also shown that its antidepressant-like effect is dependent on its interaction with the serotonergic (5-HT1A, 5-HT2A and 5-HT3 receptors), noradrenergic (α1-receptor) and dopaminergic (D1 and D2 receptors) systems. Moreover, in a more recent study, the same authors (Machado *et al.*, 2012) showed that OB mice exhibited hyperactivity and anhedonic-like behavior associated with an increased hippocampal AChE activity, parameters that were abolished by chronic treatment with rosemary hydroalcoholic extract, similar to the effects produced by fluoxetine. These results suggest that *Rosmarinus officinalis* may be further investigated as an effective therapeutic alternative for the treatment of agitated depression associated with anhedonia.

Rosemary and its biologically active compounds have also showed important anti-inflammatory properties (Altinier *et al*., 2007; Fu *et al*., 2005; Lai *et al*., 2009; Lo *et al.*, 2002; Poeckel *et al*., 2008; Scheckel *et al*., 2008). Benincá *et al.* (2011) investigated the effect of the crude extract and derived fractions of *Rosmarinus officinalis* L. on the inflammatory response in the carrageenan-induced pleurisy model in mice, confirming the anti-inflammatory properties of this plant which may be attributed, at least in part, to the presence of carnosol, betulinic acid and ursolic acid.

3.2 Volatile fraction (essential oil)

In recent years, demand for essential oils from medicinal plants has increased, particular for the oil from rosemary, on account of its widespread use as a natural food additive for food preservation thanks to its antimicrobial, antiviral, antimycotic, and antioxidant properties and, above all, its low cost and ease of availability. Rosemary oil can be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for commercial production.

Rosemary essential oil contains mainly monoterpenes and monoterpene derivatives (95–98%), the remainder (2–5%) being sesquiterpenes.

The main compounds identified in the essential oil of *R. officinalis* by GC and GC–MS analyses were reported in Figure 10.

No.	Compounds	RI	%
1	α-Pinene	934	14.9
2	Camphene	945	3.33
3	3-Octanone	966	1.61
4	Sabinene	972	0.56
5	Myrcene	982	2.07
6	O-Cymene	1013	0.71
7	1,8-Cineole	1024	7.43
8	Linalool	1089	14.9
9	Myrcenol	1104	0.75
10	Camphor	1127	4.97
11	Borneol	1155	3.68
12	Terpinen-4-ol	1166	1.70
13	α-Terpineol	1177	0.83
14	Verbinone	1187	1.94
15	Piperitone	1246	23.7
16	Bornyl acetate	1274	3.08
17	β-Caryophyllene	1424	2.68
18	*cis*-β-Farnesene	1448	1.26
19	Germacrene D	1481	0.52
20	α-Bisabolol	1673	1.01

Figure 10. Chemical composition of rosemary essential oil (Gachkar *et al.*, 2007)

Based on main compounds of essential oils, different chemotypes such as α-pinene and verbenone (Pintore *et al.*, 2002) or 1,8-cineole, verbenone and camphor

(Celiktas *et al*., 2007) were identified according to geographical and climatic regions.

The main active components are 1,8-cineole, camphor and pinene as described by Hethelyi *et al*., 1989, Panizzi *et al*., 1993, Caccioni & Guizzardi, 1994, Biavati *et al*., 1997.

The rosemary essential oil is mainly used for local applications for its balsamic, antispasmodic and anti-inflammatory activities (ESCOP monography), but the antimicrobial (Okoh *et al*., 2010; Bozin *et al*., 2007; Angioni *et al*., 2004; Kabouche *et al*., 2005), the insecticidal and larvicidal (Waliwitiya *et al*., 2009; Pavela, 2008), and the antioxidant (Bozin *et al*., 2007; Wang *et al*., 2008) properties of this essential oil are widely documented.

According to Ruberto & Baratta (2000) phenols from essential oils, in particular thymol and carvacrol, were confirmed to possess the highest antioxidant activity, compared to other compounds which have been identified. In particular some monoterpene hydrocarbons, namely terpinolene, α- and γ-terpinene showed a significant protective action, whereas among the oxygenated components, beside the aforesaid phenols, allylic alcohols manifested an appreciable activity. Sesquiterpene hydrocarbons and non isoprenoid components subjected to this study showed a low, if any, antioxidant effect.

Many researchers studied the antifungal and antimicrobial activity of the essential oil of rosemary. Baratta *et al*. (1998) tested the antibacterial and antifungal activities of a commercial sample (α-pinene, 1,8-cineole, camphor, α-terpineol chemotype) finding low activity except against *Staphylococcus aureus*. The essential oils obtained from the plant of Sardinian rosemary and their main compounds (α-pinene, (-)camphene, verbenone, bornyl acetate, camphor and borneol) showed low inhibitory activity, both against Gram (+) (*Staphylococcus aureus*, and *S. epidermidis*) and Gram (-) (*Escherichia coli*, *Pseudomonas aeruginosa*), with MIC (Minimal Inhibitory Concentration) always over 900 μL/mL (Angioni *et al*., 2004). On the other hand, the Argentinean chemotypes (myrcene/1,8-cineole/camphor) expressed insecticide properties and *in vitro* antifungal activity against *Ascosphaera apis* (Larràn *et al*., 2001).

In other study (Bozin *et al.*, 2007), the essential oil of rosemary, comparing to the bifonazole, showed lower MIC especially against *Candida albicans* and two dermatomyceta, *Trichophyton tonsurans* and *Trichophyton rubrum*, indicating its significant antifungal effect.

Masatoshi *et al*. (1997) pointed out the effectiveness of rosemary against plant parasites; both the essential oil and its major components (α-pinene/1,8-cineole/camphor) had high repellency effect against *Neotoxoptera formosana*, the onion aphid. Daferera *et al.* (2003) also evaluated the effectiveness of the essential oil (α-pinene/1,8 cineole/borneol, chemotype) from Greece on the growth of plant pathogens such as *Botritis cinerea*, *Fusarium sp.*, and *Clavibacter michiganensis*.

The essential oil composition and terpene content may be influenced by several factors.

Variations in volatile terpene composition were mostly correlated to the provenance (different geographical areas) (Chalchat *et al.*, 1993), to the environmental and agronomic conditions (Moghtader *et al.*, 2009), but also to the time of harvest (Yesil Celiktas *et al.* 2007), to the stage of development of the plants and to the extraction methods (Okoh *et al.* 2010).

In the study of Zaouali *et al*. (2010) the essential oil and polyphenol compound contents of Tunisian rosemary leaves, stems, flowers and achenes collected on branches of clonal plants were assessed, and their distribution during vegetative, flowering and fructification of branches was compared. The highest oil yield (1.43%) was obtained for leaves collected at the flowering stage and these results were in agreement with those of Chalchat & Ozcan (2008) and Aidi Wannes *et al.* (2010), reporting that these organs have the highest essential oil yield. The antioxidant activity of leaf essential oils estimated by the DPPH test system was low when compared to that of acetonic extracts. The best activity was observed for oils extracted from leaves at the flowering stage (17.75 mM Fe^{2+}), instead oils obtained from vegetative and fruiting stages exhibited a similar low activity (IC_{50} = 11.55 and 12.8 mg/ml, respectively). Furthermore essential oils of leaves, with the same length, taken at the same zone of the branches and differing by their age, were characterized by high content of 1.8-cineole (35.8%), camphor (14.5%) and α-

pinene (10.6%). Oils from stems and flowers contain high contents of caryophyllene oxide (11.4%) and β-caryophyllene (16.68%), respectively.

Beretta *et al.* (2011) also carried out an extensive study to characterize the constituents of the essential oils of the α-pinene chemotype, obtained by steam distillation of the aerial parts of the plant in the flowering, post-flowering and vegetative period, and to examine their antioxidant response. The results illustrate the difference in the antiradical and anti-lipoperoxidant activities from the aerial part of the plants in the various phases of development and show that the oil collected from rosemary during the flowering phase attained the best activity to prevent lipid oxidation and to act as biocide to combact bacterial pathogens.

3.3 Innovative extraction techniques - Green processes

Several traditional methods have been used to extract antioxidants from aromatic plants, such as conventional solvent extraction (Almela *et al.*, 2006; Doolaege *et al.*, 2007), solid–liquid extraction, aqueous alkaline extraction, extraction with vegetable oils (Señorans *et al.*, 2000), among others.

The design of more efficient extraction processes, that may address the requirements of process intensification and energy consumption reduction, has been an important research topic in recent years. Safety, sustainability, environmental and economic factors are all forcing industries to turn to non-conventional technologies and greener protocols (Chemat *et al.*, 2012).

In the last few years more environmentally friendly and selective extraction techniques have been preferred, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE).

SFE operates at low temperatures, in oxygen absence and typically using CO_2 as extraction agent; these characteristics make SFE an ideal technique for the extraction of natural antioxidants.

On the other hand, PLE is a solid-liquid extraction technique which uses organic solvents at elevated pressure and temperature in order to increase the efficiency of

the extraction process. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus enabling safe and rapid extractions.

Both techniques have been applied to antioxidants extraction from rosemary (Chang *et al.*, 2008; Señorans *et al.*, 2000; Herrero *et al.*, 2010; Linares *et al.*, 2011).

The results presented by Herrero *et al.* (2010) show the possibility to attain bioactive extracts from rosemary using these environmentally clean extraction techniques (SFE and PLE). PLE using ethanol or water as solvents at high (200 °C) or mild temperatures (100 °C) respectively, provided the best results, considering not only the higher extraction yield produced but also the amount of antioxidants extracted. In this study the performance of supercritical fluid extraction using CO_2 modified with ethanol was also assessed; this procedure has proved to be equally capable of extracting phenolic antioxidants. However, in this case, the applicability of SFE is somewhat limited given the relatively low extraction yields that this technique is able to provide. Therefore, according to this study, PLE has supplied the highest extraction yields, although SFE might be more selective and environmentally friendly.

According to Borrás-Linares *et al.* (2011), the best procedure for extracting each compound depends on its polarity. In this sense, the most polar compounds were only present in the extracts obtained by PLE using water as extracting solvent; other less polar compounds were detected in the extracts obtained by PLE using water or ethanol and on the other hand, the less polar compounds were mainly extracted by using SFE. Whereas supercritical CO_2 is a very low polarity solvent, ethanol and water (employed in PLE) allowed the extraction of more polar compounds.

With regard to UAE, this technique can be considered a green process as it helps to greatly accelerate the extraction procedure and reduce total energy consumed. The method is clean, and thanks to the low bulk temperature and the rapid execution, usually it does not degrade the extract.

It leaves no residue in the extract and uses no moving mechanical parts, preventing the occurrence of any pollution. It also offers advantages in terms of productivity, yield and selectivity, improves processing time, enhances quality, reduces chemical and physical hazards and is environmentally friendly (Chemat *et al*., 2011). In fact, to date ultrasound has been recognized for potential industrial application in the phyto-pharmaceutical extraction industry for a wide range of herbal extracts. Extraction enhancement by this technique has been attributed to the propagation of ultrasound pressure waves, and resulting cavitation phenomena. High shear forces cause increased mass transfer of extractants (Jian-Bing *et al*., 2006).

In recent years, Albu *et al*. (2004) investigated the effect of different solvents and ultrasound on the extraction of carnosic acid from rosemary. Using conventional stirred extraction, ethanol was significantly less effective then ethyl acetate and butanone. The application of ultrasounds improved the relative performance of ethanol such that it was comparable to butanone and ethyl acetate alone. Thereby ultra-sonication may reduce the dependence on a solvent and enable use of alternative solvents which may provide more attractive economics, environmental and health and safety benefits.

In recent years also microwave-assisted extraction has drawn significant research attention in various fields, in particular medicinal plant research, due to its special heating mechanism, moderate capital cost and its good performance under atmospheric conditions.

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz. In order to avoid interferences with radio communications, domestic and industrial microwaves generally operate at 2.45 GHz (Figure 11).

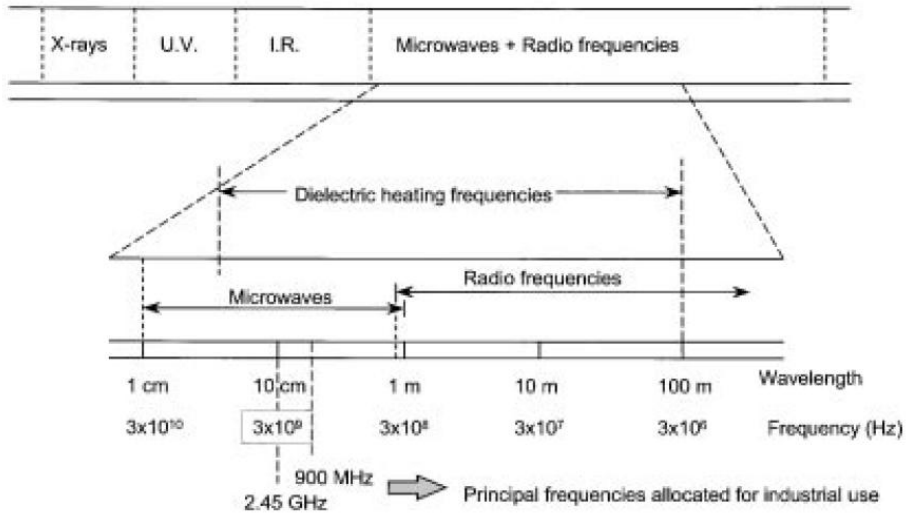


Figure 11. The electromagnetic spectrum (Kaufmann & Christen, 2002)

Owing to their electromagnetic nature, microwaves possess electric and magnetic fields which are perpendicular to each other. The electric field causes heating via two simultaneous mechanisms, namely, dipolar rotation and ionic conduction. Consequently, unlike classical conductive heating methods, microwaves heat the whole sample simultaneously (Figure 12).

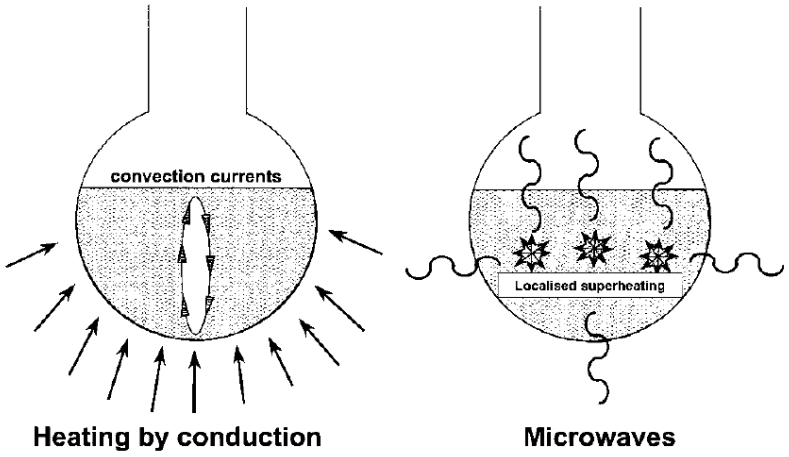


Figure 12. Scheme of the heating principle by conduction in the classical method of extraction and by microwave irradiation in MAE (Kaufmann & Christen, 2002)

The main advantage of MAE resides in the performance of the heating source. The high temperatures reached by microwave heating reduces dramatically both the extraction time and the volume of solvent required.

This technique has been continuously improved throughout the last decade where many modifications have been introduced to enhance its performance. Besides the fundamental closed system and open system of MAE, various modified MAE have been developed such as vacuum microwave-assisted extraction (VMAE), nitrogen-protected microwave-assisted extraction (NPMAE), ultrasonic microwave-assisted extraction (UMAE) and dynamic microwave-assisted extraction (DMAE). "Closed system" and "open system" are used to refer to the system that operates above atmospheric pressure and under atmospheric pressure, respectively (Dean *et al.*, 2000; Luque-García *et al.*, 2003), as illustrated in Figure 13.

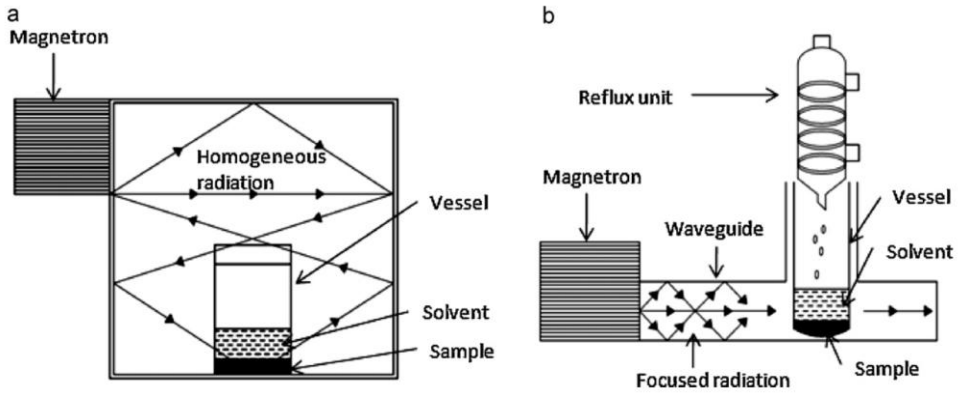


Figure 13. (a) Closed type microwave system and (b) open type microwave system (Mandal *et al.*, 2007)

In a closed MAE system, the extractions are carried out in a sealed-vessel with different mode of microwave radiations. Extraction is normally carried out under uniform microwave heating. High working pressure and temperature of the system allow fast and efficient extraction. The pressure inside the extraction vessel is controlled in such a way that it would not exceed the working pressure of the vessel while the temperature can be regulated above the normal boiling point of the extraction solvent. Despite the fact that the closed system offers fast and efficient

extraction with less solvent consumption, it is susceptible to losses of volatile compounds with limited sample throughput. Open system is developed to counter the shortcomings of closed system such as the safety issues and it is considered more suitable for extracting thermolabile compounds. This system has higher sample throughput and more solvent can be added to the system at anytime during the process. This system operates at atmospheric conditions and only part of the vessel is directly exposed to the propagation of microwave radiation (mono-mode). The upper part of the vessel is connected to a reflux unit to condense any vaporized solvent.

With all these techniques, recoveries of analytes and reproducibility are improved and, therefore, these methods should be considered as interesting alternatives and promising processes for the scale-up of plant extraction.

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OUTLINE OF THE THESIS

Plants are one of the most important sources of human foods and medicines. Rapidly increasing knowledge on nutrition, medicine, and plant biotechnology has dramatically changed the concepts about food, health and agriculture, and brought in a revolution on them. Nutritional therapy and phytotherapy have emerged as new concepts and healing systems have quickly and widely spread in recent years. Strong recommendations for consumption of nutraceuticals, natural plant foods, and the use of nutritional therapy and phytotherapy have become progressively popular to improve health, and to prevent and treat diseases. With these trends, improving the dietary nutritional values of fruits, vegetables and other crops or even bioactive components in folk herbals has become targets of the blooming plant biotechnology industry.

This PhD project attempts to remark on these aspects through the study on composition and stability of bioactive metabolites present in food and herbal products focusing, in particular, on rosemary and pigmented potatoes.

Phenolic compounds in these plant materials are closely associated with their antioxidant activity. They are also known to play important role in stabilizing lipid peroxidation and to inhibit various types of oxidizing enzymes or to act as anti-inflammatory agents.

Epidemiological studies have indicated that dietary intake of antioxidant substances from plants is inversely associated with mortality from coronary heart disease.

This antioxidant action makes the diverse groups of phenolic compounds an interesting target in the search for health-beneficial phytochemicals and also offers a possibility to use these compounds or extracts rich in them to reduce the risks of several human diseases.

The main goals of this PhD project are briefly described as following:

- Study the effect of an industrial steam cooking method by monitoring the content of anthocyanins and phenolic acids in several cultivars of potato (*Solanum tuberosum* L.) with yellow, red and violet flesh colour;
- Evaluate the efficacy of the antioxidants present in different flesh coloured potatoes after cooking, using *in vitro* antioxidant activity assay (ABTS assay), and assess their potential contribution to dietary antioxidant intake;
- Optimize an extractive and analytical procedure for the determination of all the phenolic constituents of *Rosmarinus officinalis* L. using HPLC/DAD/MS, and evaluate the chemical stability of the main phenols, depending on the storage condition, the different drying procedures and the extraction solvent;
- Optimize an innovative extraction method to recover both the volatile and the phenolic components of rosemary using a unique multistep procedure to extract the volatile terpenes and the phenolic compounds from the same sample;
- Investigate efficiency and selectivity of innovative extraction processes (ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE)), to recover the phenolic fraction from rosemary leaves by means of last-generation devices, and to verify the ability of these techniques to obtain higher yields in a very short time, comparing to traditional methods;
- Perform *in vitro* and *in vivo* tests to evaluate the potential biological effects of rosemary extracts and its main compounds, in particular:
  - the anticholinesterase property of rosmarinic acid in hyperglycaemia and its protective role against lipid peroxidation in streptozotocin-induced diabetic rats;
  - the antimicrobial activity of some chemically characterized rosemary extract against two Gram-negative bacteria, *Escherichia coli* and

Pseudomonas aeruginosa, and two Gram-positive bacteria, *Staphylococcus aureus* and *Staphylococcus epidermidis*;

- the anti-hyperalgesic effect of two phenolic rosemary extracts to inhibit neuropathic pain in the Chronic Constriction Injury (CCI) model of neuropathy.

Storage method, drying processes and extraction procedures strongly affect the phenolic fraction of rosemary leaves: an HPLC/DAD/MS study

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Keywords: Rosemary, HPLC/DAD/MS, carnosic acid, rosmarinic acid, flavonoids, chemical stability

Abstract

The *Rosmarinus officinalis* L. is widely known for its numerous applications in the food field but also for the increasing interest in its pharmaceutical properties. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction and the phenolic constituents. The latter group is mainly constituted by rosmarinic acid, by a flavonoidic fraction and by some diterpenoid compounds structurally derived from the carnosic acid. The aim of our work was to optimize the extractive and analytical procedure for the determination of all the phenolic constituents. Moreover the chemical stability of the main phenols, depending on the storage condition, the different drying procedures and the extraction solvent, have been evaluated.

This method allowed to detect up to 29 different constituents at the same time in a relatively short time. The described procedure has the advantage to being able to detect and quantify several classes of compounds, among them numerous minor flavonoids, thus contributing to improving knowledge of the plant.

The findings from this study have demonstrated that storing the raw fresh material in the freezer is not appropriate for rosemary, mainly due to the rapid disappearing of the rosmarinic acid during the freezing/thawing process. Regarding the flavonoidic fraction, consistent decrements, were highlighted in the dried samples at room temperature if compared with the fresh leaf. Rosmarinic acid, appare very sensitive also to mild drying processes. The total diterpenoidic content undergoes to little changes when the leaves are freeze dried or frozen and limited losses are observed working on dried leaves at room temperature. Nevertheless it can be taken in account that this fraction is very sensitive to the water presence during the extraction that favors the conversion of carnosic acid toward it oxidized form carnosol. From our findings, it appear evident that when evaluating the phenolic content in rosemary leaves, several factors, mainly the type of storage, the drying process and the extraction methods, should be carefully taken into account because they can induce partial losses of the antioxidant components.

1. Introduction

Rosmarinus officinalis L. (Lamiaceae) is a plant widely distributed in Europe, Asia and Africa and one of its elective growing areas is the Mediterranean basin where spontaneous plants are diffusely distributed. The plant is widely known for its numerous applications in the food field but also for the increasing interest in its pharmaceutical properties. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction and the phenolic constituents. The latter group is mainly constituted by rosmarinic acid, by a flavonoidic fraction and by some diterpenoid compounds structurally derived from the carnosic acid.

In the food industry rosemary is a very frequently used herb and its oleoresins are added to several products to improve their oxidative stability and to ameliorate the organoleptic profiles [1].

The quality and value of commercial rosemary extracts are closely related to their phenolic content, particularly of carnosic and rosmarinic acids, the most abundant constituents, which are also well known for their various biological properties. According to the ESCOP (European Society Cooperative on Phytoterapy),ethanol and aqueous extracts from rosemary leaves are used as coleretic, colagogue, epatoprotective, and antioxidants, but also as light diuretic, antiulcer, antitumor and antiviral [2] products. The derived essential oil is mainly used for local applications for its balsamic, antispasmodic and anti-inflammatory activities [2].

Moreover, rosemary and its oleoresins and extracts are widely used to preserve and improve the organoleptic and functional properties of foods. A protective effect against the discoloration of paprika samples over time was highlighted for rosemary extracts containing rosmarinic and carnosic acids [3].

The introduction of rosemary ethanol extracts to the products of wiener-type and liver sausages limited lipid oxidation better than direct addition of the antioxidant to both meat products [4]. A synergistic antioxidant effect between an extract from rosemary leaves and BHT and a synergistic interaction with BHA to inhibit *Escherichia coli* and *Staphylococcus aureus* growth were demonstrated. Therefore, rosemary not only enhances the antioxidant efficiency of BHA and BHT, but also

the antibacterial effect of BHA, allowing a decrease from 4.4- to 17-fold in the amounts of the synthetic compounds used [5].

Currently, considerable and renewed scientific interest is directed toward the rosemary plant and its various health properties. Protection exerted by carnosol against induced oxidative stress was highlighted on the liver in rats [6,7]. Anti-angiogenic potential of rosmarinic acid relating to its antioxidant properties [8] and its ability to suppress retinal neovascularization in a mouse model of retinopathy were recently pointed out [9]. These latter findings suggest this molecule could be used in the treatment of vasoproliferative retinopathies. Recently, by in vitro test on hepatic stellate cells, rosmarinic acid showed antifibrogenic effects [10]. Administration of a dose of rosemary leaf extract (200 mg/kg BW) was effective to limit weight gain induced by a high-fat diet and protected against obesity-related liver steatosis in mice [11]. Supplementing the diet with supercritical fluid rosemary extract, containing 20% carnosic acid, reduced oxidative stress in aged rats [12]. Long-term dietary administration of ground rosemary at a 1% (w/w) level in the diet improved the antioxidant status of rat tissues following carbon tetrachloride intoxication [13].

In light of all this scientific evidence, it could be of considerable interest to have a method suitable to well characterize and quantify all the phenolic constituents of the leaf and to evaluate the factors that can affect the chemical stability of these components.

The aim of our work was to improve knowledge in this field by optimizing the extractive and analytical procedure, working on natural rosemary populations from Tuscany. The efforts have been focused to develop an extractive procedure to exhaustively recover and quantify the antioxidant phenolic components, mainly rosmarinic acid, carnosic acid and its analogous, together with all the flavonoids, both from fresh and dried rosemary leaves. Within this research the chemical stability of the main phenols, depending on the storage condition, the different drying procedures and the extraction solvent, have been evaluated.

2. Materials and methods

2.1. Samples

Mature foliar tissue samples were collected from plants growing at Montebenichi (Firenze). Aliquot samples of fresh leaves were dried in an oven at 105 ◦C, by a freeze drier and at room temperature in the dark for several days. Fresh leaves were also stored at −22 ◦C for some weeks before extraction.

2.2. Extraction procedure

The leaves (5 g as fresh material and 2.5 g as dried sample were derived from 10 young twigs each of 8–10cm length) were ground in liquid nitrogen and extracted as summarized in Fig. 1. Liquid/liquid extraction (two steps) with hexane (1:1, v/v) was applied mainly to remove part of the chlorophylls. The residual ethanol solutions were directly analyzed by HPLC/DAD/ESI.

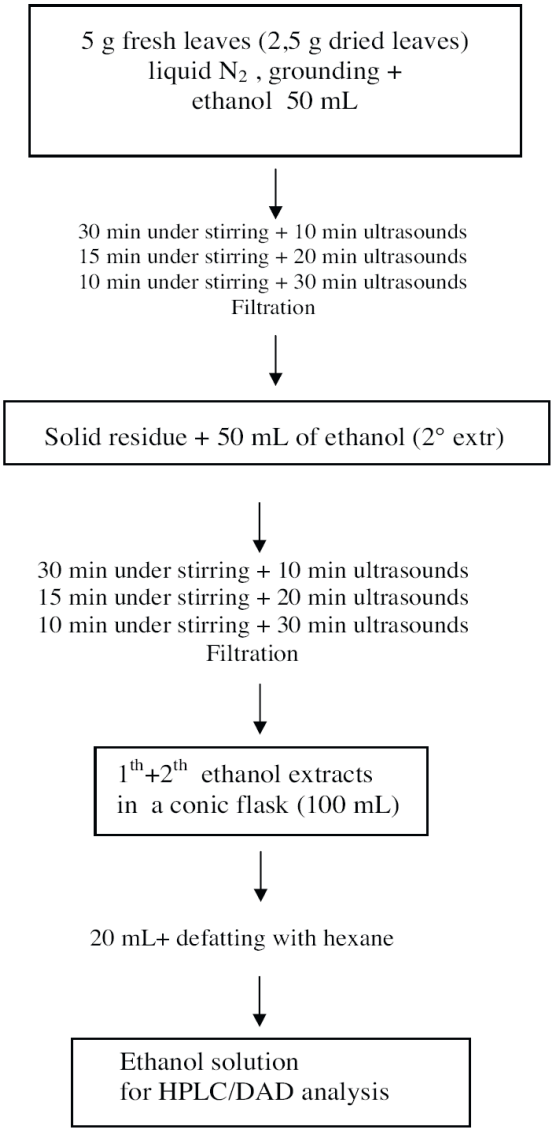


Fig. 1. Extractive scheme applied to recover the phenolic constituents of rosemary leaves. § for the extraction from dried leaves about 3% of water was added before the defatting with hexane.

2.3. HPLC/DAD/ESI analyses

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (all from Agilent Technologies, Palo Alto, CA, USA). A

150mm×3.9mm i.d., 4μm Fusion, RP18 column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN. The multi-step linear solvent gradient used was: 0–15 min 15–25% B; 15–25 min, 25–35% B; 25–35 min 35–50% B; 35–40 min 50–100% B with a final plateau of 8 min at 100%B; equilibration time 10 min; flow rate 0.4 mL min^{-1} and oven temperature 26 ◦C; injection volume 5 μL. The UV-Vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240, 284, 330 and 350 nm. After every four injections a wash method with 100% isopropanol was applied for several minutes to remove traces of liphophilic compounds from the column. The mass spectra were recorded in negative and positive ion mode, setting the fragmentation energy between 80 and 180 V and applying the same chromatographic conditions as described previously. The mass spectrometer operating conditions were: gas temperature, 350 ◦C; nitrogen flow rate, 9 L min^{-1}; nebulizer pressure, 30 psi; quadrupole temperature, 40 ◦C; and capillary voltage, 3500 V. All solvents used were of HPLC grade; CH_3CN was from E. Merck (Darmstadt, Germany).

2.4. Quantitative evaluation

The quantitative evaluation of the main constituents was performer through the use of two external standards, rosmarinic acid at 330nm and carnosic acid at 284 nm. The first compound was used at 330 nm, to quantify also all the flavonoids, while the second one at 284 nm to determine all the other diterpenoids. The calibration curve of rosmarinic acid (Sigma–Aldrich) was in a linearity range between 0.1 μg and 9.4 μg with a r^2 0.9999; the calibration curve of carnosic acid (Sigma–Aldrich) was in the linearity range of 0.05–3.4 μg with r^2 0.9998.

2.5. Statistical analyses

Data were not normally distributed (Kolmogorov–Smirnov one sample test) and were analyzed by the non-parametric Kruskal–Wallis ANOVA followed by the Mann–Whitney U-test for multiple comparisons using SYSTAT 12.0 software

(Systat Software Inc., Richmond, California, USA). Differences were accepted when significant at the 5% level.

3. Results and discussion

3.1. Extraction process

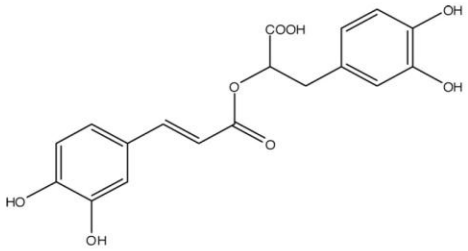
As summarized in Table 1, to recover the phenolic fraction from rosemary leaves, several procedures have been described proposing different times of extractions, solvents and weigh/volume ratios. Hydro alcoholic mixtures in various proportions [14–16], or methanol [17–20] and ethanol alone [21] have been proposed as suitable media to recover the main phenolic constituents from leaves. A small number of reports indicate the use of other solvents, such as CH_2Cl_2/EtOH, mainly for the diterpenoidic fraction [22], or DMSO [23,24], a solvent not easily to remove in case of concentration of the sample. Moreover, as highlighted in Table 1, the total amount of leaves, and the applied ratios between solvent and raw matrix varies widely, ranging between 20 mg and 10 g and between 1.1 and 100 mg/mL, respectively.

Selected amounts (f: fresh; d: dried)	Ratio (mg/mL)	Extractive mixture	Steps[a]	Compounds	References
1 g/15 mL (f)	67	CH_2Cl_2/ethanol 75:25	1	Carnosic acid, other diterpenoids, cirsimaritin, genkwanin	22
800 mg/100 mL (d)	8	Methanol	1		20
20 mg/mL (d)	20	DMSO	1	Phenolic acids and flavonoids	23; 24
50 mg/45 mL (d)	1.1	Ethanol/H_2O 3:7 and ultrasounds[b]	2	Rosmarinic and caffeic acids	14; 15
10 g/100 mL (d)	100	Ethanol and ultrasounds[c]	1	Rosmarinic and carnosic acids	21
50 mg/25 mL	2	Methanol and ultrasounds		Rosmarinic and carnosic acids, carnosol	17
500 mg/20 mL (f)	25	Methanol	4	Flavonoids, rosmarinic and carnosic acids	18
500 mg/20 mL (d)	25	Methanol	4		
1 g/45 mL (f)	22.2	Methanol	3	Flavonoids, rosmarinic, carnosic acids	19
500 mg/40 + 10 mL		Methanol/H_2O 3:7 and acidic water	1	Phenolic acids, rutin, quercetin, luteolin, rosmarinic acid	16

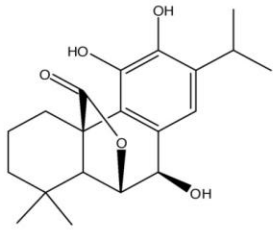
[a] Number of successive extractive steps.
[b] Ultrasounds for 10 min at 40 MHz.
[c] Ultrasounds for 45 min at 40 MHz.

Table 1. Extractive methods proposed to recover the phenolic constituents from rosemary leaves.

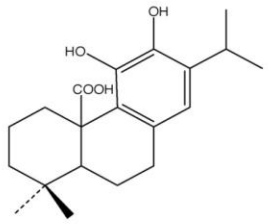
In addition, several of these studies focus only on recovering a part of the complex phenolic fraction of the leaf. In Fig. 2 are shown some chemical structures of the main phenolic constituents of this plant. To date, a unique extractive method, suitable to simultaneously and efficiently recover all these components with high yields, has not yet been proposed.



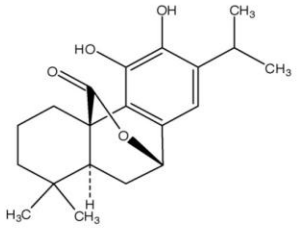
Rosmarinic Acid



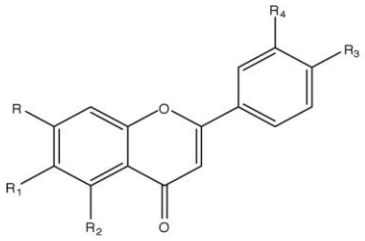
Epirosmanol



Carnosic acid



Carnosol



Esperidin-7-O-rhamnoglucoside

	R	R_1	R_2	R_3	R_4
Esperidin-rhamnoglucoside	O-glu-rha	H	OH	OCH_3	OH
Cirsimaritin	OCH_3	OCH_3	H	OH	H
Genkwanine	OCH_3	H	OH	OH	H
Luteolin O-glucoside	OH	H	OH	OH	O-glu

Fig. 2. Chemical structures of the main phenolic constituents of rosemary leaves.

With the objective of obtaining a representative sample and after some preliminary tests (data not shown), 5 g of fresh leaves and 2.5 g of dried sample were chosen to

carry out the extraction process. To select the extractive mixture, several factors were taken into account: (i) the need to use a solvent that would guarantee an adequate chemical stability of the target molecules with good extraction yields of all the components; and (ii) the willingness to use a non-toxic mixture suitable also for future applications in the food field. In light of these requirements, only ethanol and ethanol–water mixtures were chosen to carry out the extraction tests within this work. Moreover, it is worth noting that, mainly to improve the extraction yields of rosmarinic and carnosic acids, several previous works have already opted for alcoholic mixtures (Table 1).

Considering the co-presence in the leaf both of polar and non polar phenols, it was decided to first operate with ethanol mixtures containing water but in a percentage of not over 50%. Preliminary tests on fresh leaves carried out with ethanol/water (1:1) or ethanol for the first extraction and ethanol/water (1:1) for the next step, did not give encouraging results. In fact, if compared with the yields reached using only ethanol for all the extractive steps, lower amounts of rosmarinic acid and a rapid degradation of carnosic acid toward its oxidized forms, were obtained with the use of hydroalcoholic mixtures.

In literature it is reported that in water media the carnosic acid rapidly degraded toward the oxidized form of carnosol quinone [25] and in methanol it is converted toward carnosol [26] with a consistent decrease after only 24 h at room temperature [27]. Due to the lack of data in ethanol, some tests were done in this solvent to verify the stability over time not only of carnosic acid but also of the other phenols. To this purpose firstly the two standard, carnosic and rosmarinic acids, were dissolved in ethanol at different concentrations (0.29mg/mL and 0.94 mg/mL), and the solutions treated with ultrasound for more than 2 h, stored at room temperature or at −23 ◦C for different times and then analyzed by HPLC/DAD/MS. The carnosic acid remained unaltered up to 48 h at room temperature in dark so guarantying the possibility to make automated quantitative analysis by an autosampler. After 3 months at −23 ◦C the carnosic acid in ethanol solution was reduced of 17% obtaining carnosol as main oxidated derivative. Almost the same behavior was observed in ethanol extracts from rosemary leaves. The analyses of several samples

after 3 months at a −23 ◦C, highlighted increments of the oxidized derivatives in the ranges 14–18% for carnosol; with traces for rosmanol, and only a reduction up to 20% of the initial amount of carnosic acid. The other phenolic constituents of the leaves, the rosmarinic acid and the flavonoidic fraction, remained almost unaltered in ethanol solution up to 3 months at -23 ◦C.

It is known that sonication, which contributes to destroying cellular structures, guarantees a better penetration of the extractive solvent within the raw matrix. Since the rosemary leaves are fibrous and with a leathery texture, to increase the yields and to optimize the extraction time, the use of an ultrasound bath was inserted in the procedure, also according to that suggested by other authors [14,21]. Using a ultrasound bath at 35 KHz and not 40 KHz as previously applied, extraction times longer than 45 min were tested maintaining the water temperature below 50 ◦C. The coupled use of stirring and ultrasounds during the extraction has been inserted.

Due to the non-availability of an instrument able to apply, at the same time, these two conditions, it was elaborated the extraction procedure summarized in Fig. 1. This optimized procedure includes alternatively using magnetic stirring and ultrasounds with a total time of 115 min for each extraction.

Preliminary shorter times of extraction (by about half) were also applied highlighting a consistent decrease of the extractive yields. The amounts of rosmarinic acid were about four times lower and of the total flavonoids about two times lower with respect to those obtained applying longer extraction times.

The choice to use only two consecutive extractions for each fresh or dried sample was determined by the evidence that about 20–30% of the main phenolic constituents (rosmarinic acid and carnosic acid with its analogues) remained in the leaves after the first extractive step, while less than 5% of the total phenolic amount was recovered applying a third consecutive extraction.

3.2. Identification of the phenolic constituents

The analytical HPLC method was optimized with the aim to obtain a good chromatographic separation for all the numerous components of the ethanol extract.

After preliminary tests on different reverse phase columns, the choice fell on the Fusion® column. The multistep elution method was suitable to separate more than 28 different components (Fig. 3), among them the rosmarinic acid, numerous minor flavonoids, either as aglycones and glycosides, and the diterpenoidic constituents (Table 2), within a total time of 45 min.

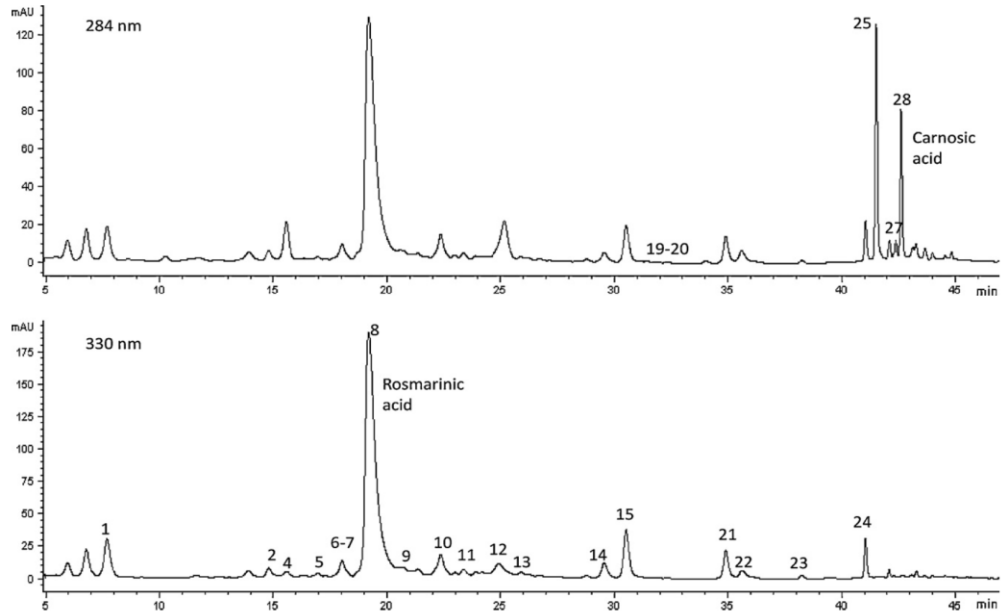


Fig. 3. HPLC profiles at 284nm and 330nm of an ethanol extracts from fresh leaves.

	Compounds	Rts	mw (aglicone)	MS (−)	MS(+)	References for flavonoids
1	Caffeic acid	7.7	180	135, 179	181	
2	Flavonoid monoglicoside	13.4	478 (316)	315, 477	317, 479	
3	Apigenin rha-glu	15.5	578 (270)			23
4	Esperidin rha-glu	15.8	610 (302)	301, 609		24
5	Diosmin rha-glu	17.4	608 (300)	299, 607		19
6	Luteolin 7-O gluc	17.9	462 (286)	285, 461		28
7	Ispidulin 7-O glu	18.5	462 (300)	461		23
8	Rosmarinic acid	18.8	360	161, 197, 359, 719		
9	Flavonoid diglicoside	20.8	640 (316)	639		
10	Cirsimaritin O-glu	22.0	476 (316)	315, 475		
11	Flavonoid diglicoside	23.0	654 (316)	653		
12	Isoscutellarein 7-O-glu	23.1	462 (286)	285, 461		23; 24
13–18	Flavonoids	24–31				
19, 20	Rosmanol/epirosmanol	34.4–35.1	346	283, 345		
21	Cirsimaritin	35.2	314	313		18; 28
22	Flavonoid	35.7			315	
23	Genkwanine	38.4	284	283	285	18; 23; 24
24	Flavonoid	41.2	–	–	329, 351	
25	Carnosol	41.6	330	285, 329, 659	331, 353	
26	4′Metoxytectochrysin	42.2	298		299	18
27	Carnosic acid derivative	42.4	374		375, 397	
28	Carnosic acid	42.8	332	287, 331		
29	Methyl carnosate	43.3	346	287, 331, 287		

Table 2. List of phenolic compounds tentatively identified by their Rts, UV–Vis and mass spectra and by the use of standards. *Glu, glucose; gluc, glucuronic acid; rha, rhamnose*.

The rosmarinic acid, easily identified by comparison with the standard, is always the main component of the phenolic fraction. Among the diterpenoids, the presence of carnosic acid was confirmed by comparison with the pure standard and a very good response of the mass detector both in negative and positive mode was highlighted. As discussed in the next paragraph, several oxidation products were obtained maintaining this standard in ethanol solution for several days; this sample was then used as reference to detect and identify the same diterpenoids in the rosemary extracts. The mass spectra of carnosic acid and carnosol resulted diagnostic both in positive and negative ionization mode as clearly shown in Fig. 4a and b.

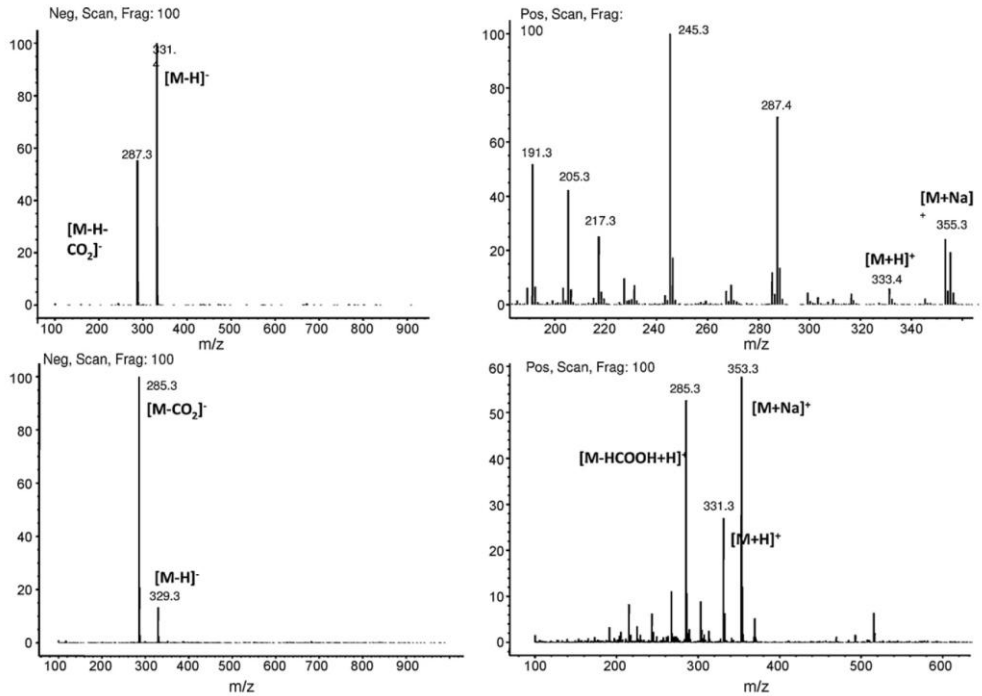


Fig. 4. Mass spectra in positive and negative ionization mode of (a) carnosic acid and (b) carnosol.

The positive ionization allowed to obtain the $[M+H]^+$ and the $[M+Na]^+$ ion species for both the compounds and the fragments related to the loss of the carboxyl group. In negative ionization mode the most intense and diagnostic ions were the $[M-H]^-$

and the $[M-H-CO_2]^-$ ion at m/z 287, and m/z 285 for carnosic acid and carnosol respectively.

In agreement with previous data that highlighted close retentions times for the two isomers, rosmanol and epirosmanol [18], and in absence of suitable pure standards, they were indentified mainly by their UV–Vis and mass spectra. Appling a negative ionization, the mass spectrum of rosmanol/epirosmanol at higher fragmentation energy, showed the $[M-H]^-$ ion at m/z 345, the $[M-H-CO_2]^-$ ion at m/z 301 and $[M-H-CO_2-H_2O]^-$ specie at m/z 283. Moreover the loss of a water molecule observed only for these compounds within the diterpenoidic fraction, can be attributed to a dehydratation process involving the free OH group linked on carbon C7. Even if in traces amount the presence of methyl carnosate was confirmed by its mass spectrum with the $[M-H]^-$ ion atm/z 345, the specie related to the loss of the methyl group atm/z 331, and the ion at m/z 287 attributable to the loss of the CH_3–COO group, typical also of the mass spectrum of carnosic acid. This metabolite was also detected, as impurity, in the commercial standard of carnosic acid.

According to literature [18,23,24,28] several flavonoidic derivatives, were detected in our extracts, even if in lower amount if compared to the main constituents, the diterpenoidic fraction and the rosmarinic acid. The applied methods allow to separate and detect an higher number of flavonoidic structures with respect to those previously reported [18,23,24,28].

These molecules were recognize to belong to the class of flavones or flavonols mainly by their characteristic UV–Vis spectra with two main bands between 260–270 nm and 335–345 nm.

Among these constituents the aglycones cirsimaritin and genkwanine showed mass spectra characterized only by the presence of the $[M-H]^-$ ions with 100% of intensity. Other glycosilated forms were also detected, among them ispidulin, cirsimaritin and isoscutellarein monoglucosides and some minor diglicosides, all showing diagnostic fragmentation in negative ionization mode (Table 2).

3.3. Effects of freezing and drying processes on the phenolic fraction

In this paragraph we discuss the main factors that strongly modify the phenolic amount in the ethanol extracts, particularly when obtained from fresh or frozen leaves. The following data were obtained working on two batches of rosemary leaves harvested from the same plants. The sample from February was selected to evaluate the consequences of freezing/thawing processes, while the other one from September was used to study the effects of drying processes.

Moreover, with the aim to propose a mode of quantization easy to be applied only the two main constituents, available as commercial standards, carnosic and rosmarinic acids, were selected as external standards; for all the flavonoids again was chosen the rosmarinic acid.

In the ethanol extract from frozen leaves the concentration of rosmarinic acid was dramatically lower with respect to the fresh leaves, and all the minor flavonoids were better highlighted. Frequently when a large number of fresh samples are handled, the sample plant material is frozen to guarantee its stability over time. For rosemary leaves it is easy to observe a rapid and marked browning of the surface of the leaf during thawing. In fact, even if the frozen sample had been handled within a few minutes (e.g. not more than 5min), before the addition of the extractive solution a partial browning could not be avoided and a significant decrease of rosmarinic acid (χ^2= 3.857, $P < 0.05$) between 65% and 80% was measured (Table 3). This phenomenon must be taken into account when a quantitative determination of this phenol in rosemary leaf is done. The enzymatic browning can be related to the endogenous enzymes that, during and after the thawing, presumably oxidize the two catecolic groups of rosmarinic acid. This aspect has not yet been sufficiently emphasized in the scientific literature to date. In fact, despite these problems, freezing has been recently reported as a method to store, over time prior to chemical analysis, aromatic plants including rosemary [16]. In agreement with our results, these authors observed that the antioxidant activity of all the extracts from frozen leaves was considerably lower when compared with those from dried leaves. This evidence can be mainly related to the oxidation of rosmarinic acid.

	Fresh		Frozen		
	µg/g	SD	µg/g	SD	
Flavonoid monoglu.	205.15	17.51	189.94	3.57	NS
Esperidin rha-glu	102.11	8.71	113.15	2.36	NS
Ispidulin 7-O-gluc	420.01	22.09	365.68	21.42	*
Rosmarinic acid	12349.83	1321.14	3520.84	604.67	*
Cirsimaritin glu.	573.93	47.57	473.91	41.60	*
Flavonoid diglu	116.56	5.44	82.74	3.09	*
Isoscutellarein 7-O-glu	444.51	14.15	474.43	24.27	NS
Flavonoid	109.03	7.14	94.59	2.36	*
Flavonoid	58.77	1.09	50.78	3.22	*
Flavonoid	376.66	9.30	326.51	31.24	*
Flavonoid	1088.47	57.51	828.49	56.22	*
Cirsimaritin	581.47	70.19	525.97	14.86	NS
Flavonoid	202.63	40.90	167.78	16.14	NS
Genkwanine	88.54	3.30	81.71	2.36	NS
Flavonoid	423.15	34.29	461.03	13.86	*
Total flavonoids	4790.98	280.2	4236.71	186.16	*
Carnosolo	11283.96	129.22	12894.43	872.79	*
Carnosic acid	14240.19	253.06	14562.51	338.33	NS
Total Terpenoids	25524.15	273.95	27456.94	1030.27	NS
Total phenols	42664.97	1372.8	35214.49	1419.89	*

* $P < 0.05$.

Table 3. Amount of phenols (µg/g dried weight) for fresh and frozen leaves harvested in February. The values are a mean of three different extractions of the same batch of an adult plant.

At the same time, in the extract from frozen leaves also the total flavonoidic content decreased significantly but not more than 11.5% (Table 3). The extracts from fresh and frozen leaves did show only little differences in the terpenoidic fraction, and the relative distribution within the different constituents was almost unchanged as well (Fig. 5).

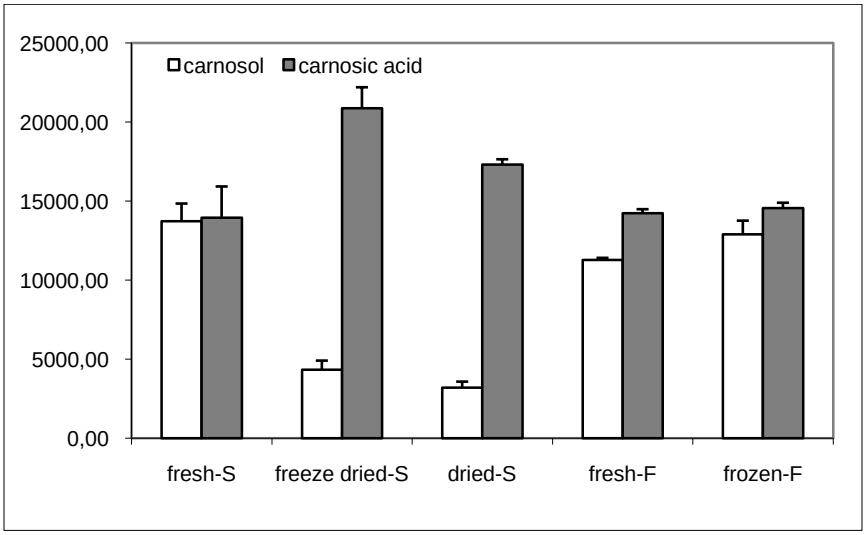


Fig. 5. Distribution of the different diterpenoids in ethanol extract from fresh, dried and frozen leaves. The data, expressed as µg/g dried leaves, are a mean of three determinations. S = leaves harvested in September; F = leaves harvested in February.

Due to the loss of phenols induced by freezing of the leaves, drying processes under mild conditions were hypothesized as alternative methods to maintain the raw sample over time. Toward this aim two procedures were tested: freeze drying and drying at room temperature for several days in the dark. The optimized extraction procedure was then applied to evaluate the phenolic content in dried and freeze-dried leaves with respect to the fresh sample, all obtained from the same batch of adult Tuscan plants (Table 4).

	Freeze					
	Fresh	SD	Freeze Dried	SD	Dried	SD
Flavonoid monoglu	964.40c	40.12	589.55b	3.39	420.99a	58.39
Esperidin rha-glu	605.71	38.81	437.64	5.33	456.41	60.74
Ispidulin 7-O-gluc	1298.29c	92.59	674.46b	21.47	514.02a	7.99
Rosmarinic acid	6611.61b	1069.85	6302.35b	183.29	3214.24a	319.25
Cirsimaritin glu.	1137.07	24.77	540.90	14.54	512.74	66.57
Flavonoid diglu	1332.95c	109.86	426.54b	1.96	387.28a	0.00
Isoscutellarein 7-O-glu	597.12c	4.42	462.81b	7.13	414.59a	1.48
Flavonoid	600.16	8.49	467.51	2.66	479.45	22.65
Flavonoid	549.08c	9.93	409.90b	0.74	NQ	–
Flavonoid	649.85c	16.83	429.53b	4.43	NQ	–
Flavonoid	1563.78c	84.04	711.16b	22.65	387.28a	0.00
Cirsimaritin	1399.63b	60.55	1213.40b	59.07	1059.78a	36.08
Flavonoid	591.82	8.77	566.08	6.44	549.86	11.16
Genkwanine	730.65	28.44	675.74	33.24	638.62	13.57
Flavonoid	1291.09	79.48	1197.18	81.31	1055.94	19.26
Total flavonoids	13311.58c	509.77	8802.39b	208.01	6255.17a	75.78
Carnosol	13717.45c	1131.95	4328.34b	586.50	3190.83a	394.04
Carnosic acid	13951.31a	1977.71	20863.83c	1341.36	17307.04b	343.71
Total Terpenoids	27668.77	2810.73	25192.17	870.14	20497.86	67.28
Total phenols	47591.96c	4164.01	40296.9b	1242.02	29967.28a	400.3

Table 4. Amount of phenolic constituents (μg/g dried weight) in fresh, freeze dried and dried leaves harvested in September. The values are a mean of three different extractions of the same batch of an adult plant. Different letters mean significant differences between the samples based on Mann–Whitney test.

Observing the quantitative results, particularly those summarized in Table 3, an high variability of the phenolic content is pointed out. Nevertheless, also some recent works, focused to evaluate these compounds in fresh rosemary leaves, often highlight an high variation of the quantitative data. Almela et al. [18] have shown RSD values up to 14% for rosmarinic acid, 25.6% for carnosol and 18.6% for carnosic acid. Other authors, even applying a completely different extraction procedure such as a supercritical fluid extraction, have had similar findings, with RSDs between 3.01% and 13.5% for rosmarinic acid [29]. Differently from the fresh leaves, the data related to the dried samples, particularly for the freeze dried leaves (Table 4) have shown lower variability.

The rosmarinic acid content did not vary significantly after lyophilization, while Mann–Whitney U-test showed a significant variation between fresh and dried leaves ($\chi^2 = 4.257$, $P < 0.05$) and between freeze-dried and dried leaves ($\chi^2 = 3.857$, $P < 0.05$). The total flavonoids showed a significant decrease, close to 34% and 53% in the freeze-dried and dried leaves respectively, with the highest losses observed for some glycosilated forms. The total diterpenoidic content did not show significant variations between the different treatments but the contents of carnosol and carnosic acid strongly varied. The highest amount of carnosol was detected in fresh leaves and Mann–Whitney U-test showed significant variations between fresh and dried leaves, freeze-dried and dried leaves and fresh and freeze-dried leaves (Table 4). Fresh leaves showed the lowest content of carnosic acid and significant variations were detected between fresh and dried leaves ($\chi^2 = 3.857$, $P < 0.05$), freeze-dried and dried leaves ($\chi^2 = 3.857$, $P < 0.05$) and fresh and freeze-dried leaves ($\chi^2 = 3.857$, $P < 0.05$) as also showed in Fig. 5.

Our previous results obtained on leaves collected in different periods confirmed this trend: the carnosic acid/carnosol ratio ranged between 0.6 and 1.8 for the fresh sample, while it increased up to 4.9–8.4 for dried samples. This phenomenon can be related to the higher water content in the fresh leaves, close to 60% (w/w) in our samples. Previous data pointed out that the water content of fresh rosemary leaves ranges usually between 40–60% by weight, while the dried matrix contains only a residual humidity near 5% (w/w) [21]. The moisture of the fresh sample can modify, particularly during the first extractive step of our method, the composition of the extractive mixture reaching a concentration near 6% (v/v). In light of our results, it appeared that also these low amounts of water seems to be critical to induce the oxidation of carnosic acid toward carnosol during the extraction process.

The water content of the sample strongly influence the variabilità of the data: working on other rosemary dried leaves, also with very low content of phenolic constituents, the RSD values obtained from freeze dried samples were always below 5.14% (data not shown).

Furthermore, these preliminary findings pointed out that the phenolic profiles in fresh foliar tissue remain almost stable between samples collected on September

and February (Table 4), with the only exceptions being the total flavonoids that showed an higher amount in the sample of September (Fig. 6). Within this class, almost all the flavonoids showed significant variation between these two sampling dates, particularly all the glycosilated forms and particularly the genkwanine is reduced up to eight times in the sample from February.

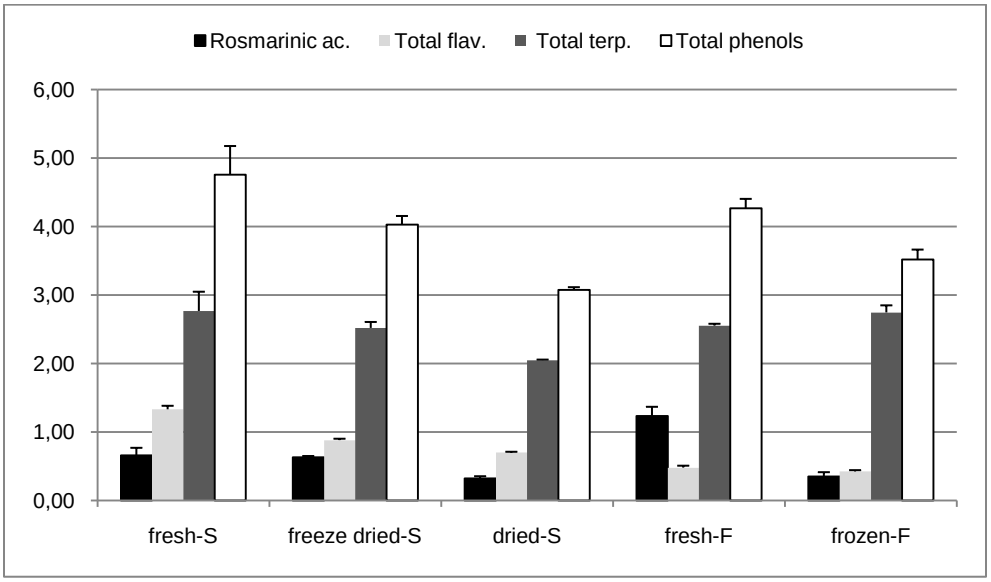


Fig. 6. Percentage values of the phenolic constituents in dried leaves after different treatments. Data are a mean of three determinations. S = leaves harvested in September; F = leaves harvested in February.

The leaves harvested in September, richer in flavonoidic compounds with respect to those collected in February (see Tables 3 and 4), can be used for further researches to isolate and characterize the minor unknown flavonoids of rosemary.

4. Conclusions

When it is impossible to extract the fresh material and to limit losses of the phenolic content, the freeze dried process is often applied as the best method for storing the sample before analysis. The findings from this study have demonstrated that storing the raw fresh material in the freezer is not appropriate for rosemary, mainly due to

the rapid disappearing of the rosmarinic acid presumably involving the phenoloxidase activity. Further research is needed to evaluate the roles of these endogenous enzymes in determining the oxidation of this compound during the freezing/thawing process. Regarding the flavonoidic fraction, consistent decrements, were highlighted in the dried samples at room temperature if compared with the fresh leaf. Differently from rosmarinic acid, limited losses were pointed out in the extracts from frozen leaves. Rosmarinic acid, appeared very sensitive also to mild drying processes. Regarding the total diterpenoidic content this undergoes to little changes when the leaves are freeze dried or frozen and limited losses are observed working on dried leaves at room temperature. Nevertheless it can be taken in account that this fraction is very sensitive to the water presence during the extraction that favors the conversion of carnosic acid toward it oxidized form carnosol. From our findings, it appear evident that when evaluating the phenolic content in rosemary leaves, several factors, mainly the phenoloxidases activity in the fresh and frozen leaf, the drying process and the extraction methods, should be carefully taken into account because they can induce partial losses or modification of the antioxidant components.

It has been pointed out, that the highest variation of the quantitative data is related to the treatment of fresh leaves and to the role of endogenous oxidative enzymes in presence of water, while this problem does not exist working on dried leaves. Despite the variability observed in the fresh samples, the statistic evaluation has pointed out, in any case, some macroscopic differences as statistically significant.

Moreover the proposed HPLC method has the advantage to detect up to 30 phenolic constituents belonging to different chemical classes. At the same time, it allows the quantitative evaluation of numerous compounds, among them several minor flavonoids. The method guarantees an analytical procedure suitable for detecting all the constituents that contribute to the antioxidant potency of the rosemary extracts in a relatively short time.

Acknowledgments

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Rosmarinic acid prevents lipid peroxidation and increase in acetylcholinesterase activity in brain of streptozotocin-induced diabetic rats

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Abstract

We investigated the efficacy of rosmarinic acid (RA) in preventing lipid peroxidation and increased activity of acetylcholinesterase (AChE) in the brain of streptozotocin-induced diabetic rats. The animals were divided into six groups (n=8): control, ethanol, RA 10 mg/kg, diabetic, diabetic/ethanol and diabetic/RA 10 mg/kg. After 21 days of treatment with RA, the cerebral structures (striatum, cortex and hippocampus) were removed for experimental assays. The results demonstrated that the treatment with RA (10 mg/kg) significantly reduced the level of lipid peroxidation in hippocampus (28%), cortex (38%) and striatum (47%) of diabetic rats when compared with the control. In addition, it was found that hyperglycaemia caused significant increased in the activity of AChE in hippocampus (58%), cortex (46%) and striatum (30%) in comparison with the control. On the other hand, the treatment with RA reversed this effect to the level of control after 3 weeks. In conclusion, the present findings showed that treatment with RA prevents the lipid peroxidation and consequently the increase in AChE activity in diabetic rats, demonstrating that this compound can modulate cholinergic neurotransmission and prevent damage oxidative in brain in the diabetic state. Thus, we can suggest that RA could be a promising compound in the complementary therapy in diabetes.

Keywords: streptozotocin, diabetes, lipid peroxidation, acetylcholinesterase, rosmarinic acid

1. Introduction

Diabetes mellitus, a major crippling disease refers to the group of diseases that lead to high blood glucose levels resulting from either low levels of the hormone (insulin) or from abnormal resistance to insulin's effects.[1] The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030.[2] During diabetes, persistent hyperglycaemia causes the increased production of free radicals, as a result of glucose auto-oxidation and protein glycosylation.[3,4] High level of lipid peroxidation has been found in diabetic patients.[3,4] Peroxidation of membrane lipids seriously impairs membrane functions and disturbs ionic gradient receptor and transport functions, results in cellular dysfunctions.[5,6] In addition, increased thiobarbituric acid reactive substances (TBARS) in rats with streptozotocin (STZ)-induced diabetes is a well-established method for monitoring lipid peroxidation.[7]

It has been observed that reactive oxygen species contribute to the development of chronic complications in the brain.[8,9] Along with cerebrovascular disease, diabetes is implicated in the development of other neurological co-morbidities including alterations in neurotransmission, electrophysiological abnormalities, structural changes and cognitive dysfunction.[10] Furthermore, diabetes has been implicated as a risk factor for dementia not only of vascular type but also to Alzheimer's disease.[11] The exact pathophysiology of cognitive dysfunction and cerebral lesions in diabetes is not completely understood, but it is likely that hyperglycaemia, vascular disease, hypoglycaemia, insulin resistance and oxidative stress play significant roles.[12] Furthermore, some research using investigational diabetes established an increase in acetylcholinesterase (AChE) (3.1.1.7) activity, which may lead to alterations in cholinergic neurotransmission and thus be associated to cognitive impairments observed in diabetes mellitus.[13] Researches have long-established that one of the most vital mechanisms responsible for correct cholinergic function is performed by the enzyme AChE.[14,15]

Acetylcholinesterase is a membrane bound enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) into choline and acetate after their function in

cholinergic synapses at the brain region.[16,17] The AChE is present in higher amount in healthy human brain compare with other tissues of the body.[16] Interestingly, AChE responds to various insults including oxidative stress, an important event that has been related to the pathogenesis and progression of a variety of CNS disorders, such as stroke, Alzheimer's disease,[11] and diabetes mellitus.[13,14,18]

Literature reveals the role of antioxidants and suggests that there is a strong association between high intake of antioxidants and low incidence of diseases linked with free radicals such as diabetes.[19,20] It has been proved that plants are the source of compounds with antioxidant properties.[21] This activity is mostly related to phenolic compounds such as rosmarinic acid (RA) (Figure 1).[22] It is a well-known natural product found in rosemary (*Rosmarinus officinalis*), lemon balm (*Melissa officinalis*) and other medicinal plants such as thyme, oregano, savoury, peppermint and sage.[24–26] Studies showed that RA is a strong antioxidant than Trolox and Vitamin E and has attributed the this propriety the most of its beneficial effects.[26] Moreover, it has many biological effects such as antitumor, anti-hepatitis and protecting the liver, inhibiting the blood clots and anti-inflammation.[24–26]

Interestingly, previous studies of our research group also demonstrated that polyphenols, such as resveratrol, prevent the increase in AChE as well the increase in lipid peroxidation.[15,27] However, the effects of RA in these parameters still were not determined. Thus, the principal aim of the present study was to evaluate anticholinesterase property of RAs in hyperglycaemia and its protective role against lipid peroxidation in STZ-induced diabetic rats.

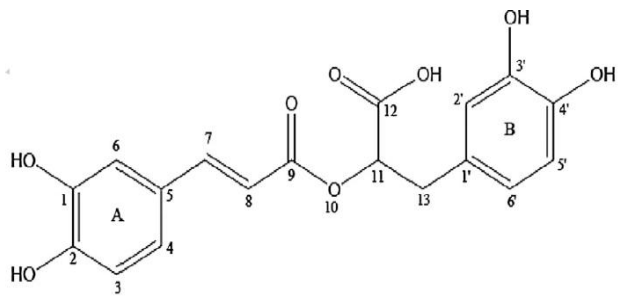

Figure 1. Chemical structure of rosmarinic acid

2. Materials and methods

2.1. Chemicals

Coomassie brilliant blue G-250, 5,5′dithiobis-2-nitrobenzoic acid, acetylthiocholine iodide (AcSch) and RA were kindly gifted by Nadia Mulinacci from Italy. STZ was obtained from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in experiments were of analytical grade.

2.2. Animals

Adult male Wistar rats (70–90 days; 200–250 g) were used in experiment obtained from Central Animal House of the Federal University of Santa Maria, Brazil. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12-h light/dark cycle with free access to food and water. Before starting the experiment, the animals were gone through an adjustment period of 20 days. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 023/2012).

Experimental induction of diabetes

Diabetes was induced by a single intra-peritoneal injection of 55 mg/kg STZ, diluted in 0.1M sodium-citrate buffer (pH 4.5). The age-matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hyperglycaemic shock. Blood samples collected from the tail vein 8 days after STZ induction. Glucose levels were measured with a portable glucometer (ADVANTAGE, BoehringerMannheim, MO, USA). Only animals with fasting glycaemia over 300mg/dL were considered diabetic and used for the present study.

Treatment

The animals were randomly divided into six groups (eight rats per group):

1. control;
2. ethanol;
3. RA 10 mg/kg;
4. diabetic;
5. diabetic/ethanol
6. diabetic/RA 10 mg/kg.

Two weeks after diabetes induction, the animals belong to the group control/RA and diabetic/RA received 10 mg/Kg body weight of RA, whereas the animals from control and diabetic groups received saline solution. RA prepared freshly in 25% ethanol and administered via gavage, between 10 and 11 AM once a day during 21 days, at a volume not exceeding 0.1 mL/100 g rat weight. The choice of this dose of 10 mg/Kg of RA was made on the basis of previous works that used the same concentrations of RA and obtained beneficial results.[28,29]

Rosmarinic acid was dissolved in 25% ethanol. To correct the interference of ethanol, a group of control rats and another group of diabetic rats received a solution of ethanol 25%. However, no significant differences in the control/ethanol and diabetic/ethanol groups were observed to any parameters analysed when compared with control/saline and diabetic/saline groups, respectively (data not shown).

Twenty-four hours after the last treatment, the animals were previously anaesthetized for blood collection by cardiac puncture, and the liver, kidney and brain were removed carefully for subsequent biochemical analysis.

Brain tissue preparation

The animals were anaesthetized under halothane atmosphere before being killed by decapitation and brains were removed and separated into cerebral cortex, hippocampus, striatum, cerebellum and placed in a solution of 10mM Tris–HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at -8 °C

until utilization. Protein was determined in each structure: cerebral cortex (0.7mg/mL), striatum (0.4 mg/mL) and hippocampus (0.8 mg/mL).

Protein determination

Protein in different structure of rat's brain was determined by the method of Bradford et al. (1976)[30] using bovine serum albumin as a standard solution.

Determination of lipid peroxidation

Lipid peroxidation in brain hippocampus and cortex was determined according to Ohkawa et al. (1979).[31] The amount of TBARS was expressed as nanomoles malondialdehyde per milligrams tissue.

Determination of AChE activity

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellmann et al. (1961)[32] as previously described by Rocha et al. (1993).[33]

The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acidnitrobenzoic, measured by absorbance at 412 nm during 2 min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM AcSCh. All samples were run in duplicate or triplicate, and the enzyme activity was expressed in micromole AcSCh/h/mg of protein.

Statistical analysis

Statistical analysis was performed by the commercial SPSS package for Windows©. All data were expressed as mean ± SD. Data were analyzed statistically

by a two-way analysis of variance followed by the Duncan's multiple tests. Differences were considered significant when the probability was $P<0.05$.

Results

The body weight and blood glucose levels determined at the onset and at the end of the experiment are presented in Table 1. As can be observed, the blood glucose levels in the diabetic group treated with RA (10mg/Kg body weight/day) for 21 days showed no significant differences from diabetic group (Table 1), whereas the body weight was significantly decreased in diabetic group compared with control. Furthermore, diabetic group treated with RA increased the body weight compared with diabetic (Table 1).

Despite that behaviour analysis is not performed, it was possible to observe that diabetic animals presented apathetic and lethargic characteristics when compared with control group. However, the diabetic rats treated with RA presented an apparent improvement in these characteristics when compared with those not treated.

Diabetic rats showed an increase in the levels of lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) when compared with control. The treatment with RA prevented the increased in the lipid peroxidation in hippocampus (28%), cortex (38%) and striatum (47%) of diabetic rats when compared with the control (Figures 2–4).

In the present study, diabetic rats presented an increase in the AChE activity in the hippocampus (58%) (Figure 5), cortex (46%) (Figure 6) and striatum (30%) (Figure 7) when compared with the control group. On the other hand, the treatment with RA (10 mg/kg body weight/day) by a period of 3 weeks prevented the increase in AChE activity in hippocampus, cortex and striatum, (Figures 5–7) compared with diabetic/saline group.

Discussion

Hyperglycaemia is the main reason of causing a series of biochemical events, which result in the formation of high levels of reactive oxygen species and ultimately an oxidative stress.[34]

Groups	Glucose (mM)		Body weight (g)	
	Onset	End	Onset	End
Control	120 ± 10.10	110 ± 8.06	266 ± 4.50	284 ± 8.15
RA	126 ± 8.86	132 ± 6.85	267 ± 5.19	299 ± 9.40
Diabetic	460 ± 20.28*	478 ± 19.32*	250 ± 5.09	189 ±15.44*
Diabetic/RA	502 ± 32.13*	502 ± 23.56*	200 ± 4.17	262 ± 7.47*

RA, rosmarinic acid. Values are expressed as mean ± SD. *Significant difference when compared with the control/saline and rosmarinic acid groups (*$P < 0.05$, $n = 6$ and 8). Analysis of variance Duncan's test.

Table 1. The effect of different doses of rosmarinic acid on body weight and fasting blood glucose levels in control and diabetic rats at the onset and the end of the experiment

STZ-induced diabetes is a well-characterized experimental model for type 1 diabetes due to its ability to selectively destroy pancreatic islet of β-cells leading insulin deficiency and hyperglycaemia.[35] In STZ-induced diabetic rats, a decreased body weight was observed (Table 1). There are different views about this loss of weight; for example, it may be related to excessive break-down of tissue proteins[36] or dehydration and catabolism of fats and proteins.[37] RA administration to diabetic rats decreased food consumption and improved body weight.

Free radicals react with important biological molecole (nucleic acids, proteins and lipids etc.). However, the most vulnerable ones are polyunsaturated fatty acids. Reaction of free radicals with cell membrane constituents leads to lipid peroxidation.[35] In our study, an increased of lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) was observed in diabetic rats as evidenced by increase in TBARS levels. This increased in lipid peroxidation levels during the diabetes can be due to inefficient anti-oxidant system.[39] In fact, several studies have demonstrated a decrease in antioxidant enzymes, such as superoxide

dismutase and catalase and consequently in increase TBARS levels, in brain of diabetic rats, which can contribute to oxidative damages in central nervous system[40,41] and consequently results in development and progression of several neurodegenerative disease. Furthermore, it is reported that high level of lipid peroxidation is responsible for the formation of lipid hydroperoxides in membrane, which result in the damage of membrane structure and alteration of membrane-bound enzymes such as AChE.[42]

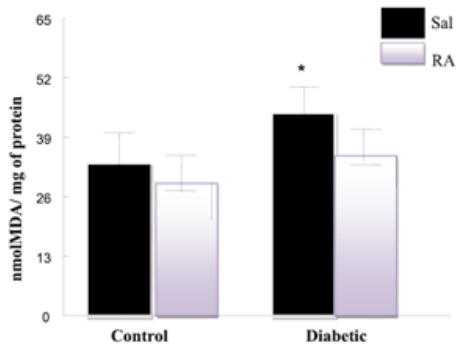

Figure 2. Protective role of rosmarinic acid in streptozotocin-induced diabetic rats via inhibition of lipid peroxidation in hippocampus. Rosmarinic acid was given by gavage for 3 weeks at the rate of 10 mg/Kg body weight. The result represents the mean of eight different experiments of each group down in duplicate. *P<0.05, diabetic group shows significant difference from all groups

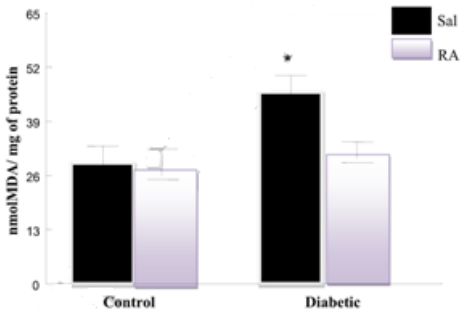

Figure 3. Lipid peroxidation in streptozotocin-induced diabetic rats in cortex and those treated with rosmarinic acid (10 mg/Kg) after 3 weeks. The results represent the means of eight different experiments down in duplicate. *P<0.009, shows significant difference from all groups

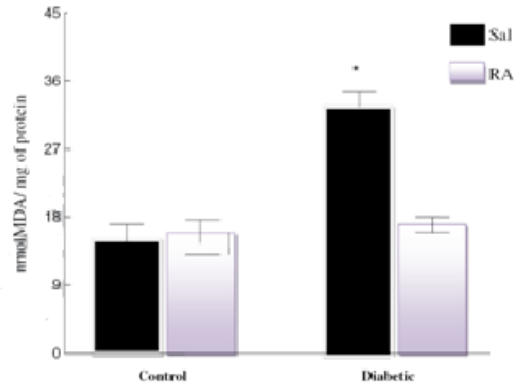

Figure 4. Lipid peroxidation in rat hippocampus after 21 days treatment with rosmarinic acid at 10 mg/Kg. The diabetic groups indicate significant (*P<0.0009) difference from all groups. The results represent the mean of eight different experiments of each group down in duplicate

In the present study, we found a significant high activity of AChE in hippocampus (Figure 5), cortex (Figure 6) and striatum (Figure 7) of STZ-induced diabetic rats compared with control group. Similarly, Schmatz et al. (2009)[15] and Sanchez-Chavez Salceda (2000)[43] also observed a significant elevation in AChE activity in cerebral cortex, striatum and hippocampus of STZ-induced diabetic rats. Interestingly, AChE activation leads to a fast ACh degradation and a subsequent downstimulation of ACh receptors causing undesirable effects on cognitive functions.[44] In this context, we can suggest that the increase in AChE activity caused by experimental diabetes leads to a reduction in the efficiency of cholinergic neurotransmission due to a decrease in Ach levels in the synaptic cleft, thus contributing to the progressive cognitive impairment and other neurological dysfunctions seen in diabetic patients.[45] On the other hand, ACh is considered as an anti-inflammatory molecule, and a possible reduction in their levels due to the increase on the AChE activity can contribute to increase the levels of interleukin-1 and tumour necrosis factor-α due to anti-inflammatory role exerted by this neurotransmitter.[16] All these events can lead to enhance local and systemic inflammations.[16,46] In fact, diabetes mellitus and Alzheimer's disease share a

common feature of low-grade systemic inflammatory conditions in which plasma AChE activity is increased.[45,46]

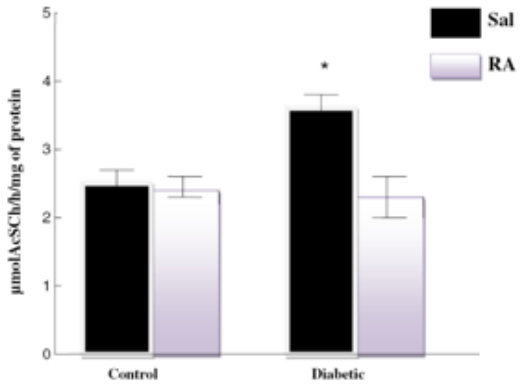

Figure 5. In hippocampus, acetylcholinesterase activity levels in streptozotocin-induced diabetic rat model and treated with rosmarinic acid (mean ± SD, n = 8). Significant differences from other groups (*P≤0.05)

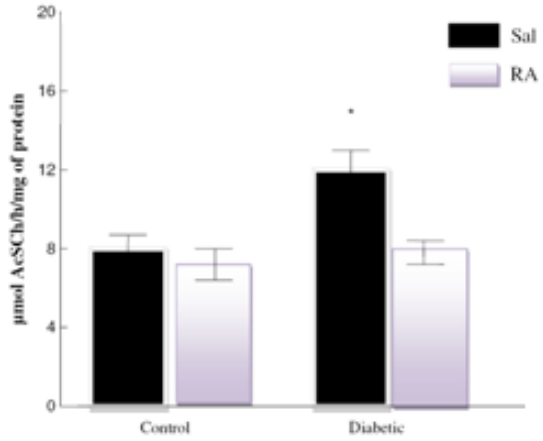

Figure 6. Acetylcholinesterase activity in cortex of STZ-induced diabetic rats and those treated with rosmarinic acid (10 mg/kg body weight) after 3 weeks treatment. Bars represent means ±SEM *P<0.001, significant increase compare with other groups

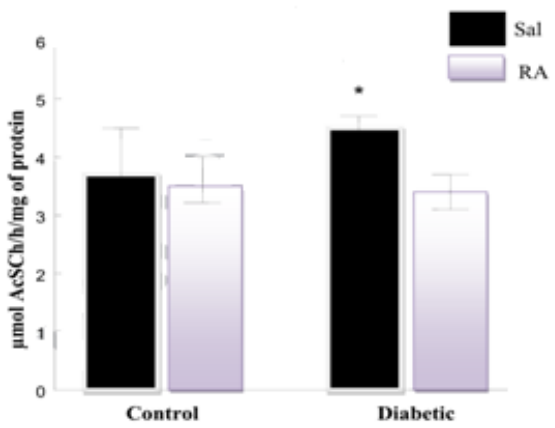

Figure 7. Acetylcholinesterase activity in striatum of streptozotocin-induced diabetic rats and those treated with rosmarinic acid (10 mg/kg body weight) after 3 weeks treatment. Bars represent means ±SEM *P<0.05, significant increase compare with other groups

Treatment of diabetes mellitus and its complications in the recent context has focused on the usage of naturally occurring antioxidants in food or medicinal flora to replace synthetic antioxidants, which are being restricted, due to their adverse side effects, such as carcinogenicity.[47] Several studies had shown that plants are source of compounds with antioxidant property and prevent lipid peroxidation in various tissues during induced oxidative stress. The activities are mostly related to phenolic compounds.[48,49]

In the present study, RA decreased lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) of diabetic rats. These results are in accordante with other studies that have showed the antioxidant effects of RA, reducing the levels of malondialdehyde in central nervous system.[50]

An important aspect to be discussed in our study is that the prevention of increase of TBARS levels by RA can be associated with the anticholinesterase property exhibit by this polyphenol. In fact, the treatment with RA prevented the increase in AChE activity of hippocampus (Figure 5), cortex (Figure 6) and striatum (Figure 7) of diabetic rats after 21 days of treatment. These results are similar to those found in studies with other antioxidants polyphenols that also prevented the rise in AChE activity. This effect in AChE enzyme can contribute to increase the ACh levels in

the synaptic cleft enabling an improvement in cognitive functions, such as learning and memory,[51] which suggests an interaction between RA and the cholinergic system. On the other hand, it is important to point out that the protective effects against oxidative stress by decreasing lipid peroxidation in brain of diabetic rats as observed in the treatment with RA could be a decisive factor to the prevention of alteration in AChE activity. In fact, alterations in the lipid membrane observed during the diabetic state can be directly associated with modification of the conformational state of the AChE molecule and would explain the change activity of this enzyme in diabetic state.[52]

In conclusion, the results obtained in the present study demonstrate an increase in lipid peroxidation in brain from diabetic rats that were associated with alterations in AChE activity indicating that cholinergic neurotransmission is altered in the diabetic state. In addition, the treatment with RA prevented the increase in AChE activity and of lipid peroxidation, demonstrating that this compound may modulate cholinergic neurotransmission and may consequently improve cognitive dysfunctions associated to oxidative stress.

Thus, we can suggest that RA is a promising natural compound with important neuroprotective actions, which should be investigated in future studies in order to find a better therapy for patients with cholinergic disorders caused by the hyperglycemic state.

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Flesh coloured potatoes as promising source of strong antioxidant compounds

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Abstract

Potatoes are rich in secondary metabolites which can play an important role as bioactive molecules in the human diet, but these compounds can be destroyed or altered during the cooking process. This study aims at evaluating the effect of an industrial steam cooking method by observing the content of anthocyanins and phenolic acids in several cultivars of potato with yellow, red and violet flesh colour. From the quantitative point of view, significant variations were pointed out in the concentration of the phenolic compounds in the steam boiled tubers. The content of total phenolic acids was determined for eight colored varieties and ranged from 38.14 to 1153.02 μg/g of fresh material (FM) in fresh potatoes and from 114.32 to 1352.1 μg/g FM in cooked potatoes. An increment was observed for six cultivars after boiling (Mz064, Vitelotte Noir, Mz012, Mz032, Mz080, Mz046). The anthocyanin content decreased in relation to the cultivar and only in one sample (Mz012) an increment was evidenced. The antioxidant activity, using ABTS assay, was also estimated on the cooked tubers. Yellow-fleshed cultivars have shown lower antioxidant activity (3,25 times lower than the violet-fleshed tubers) and a positive correlation was found between their activity and content of phenolic acids.
For red and purple-fleshed potatoes a strong correlation between antioxidant activity and total anthocyanin content was observed, suggesting that these compounds are responsible mostly for the antioxidant properties.

Keywords: *Solanum Tuberosum*, pigmented potatoes, anthocyanins, chlorogenic acid, cooking process, ABTS test

1. Introduction

Potatoes, belonging to the species *Solanum tuberosum* L., constitute a food highly popular worldwide prepared and served in a variety of ways. Because of high contents of carbohydrates, proteins and vitamin C potatoes belong to the staple food in many regions of the world.

Freshly harvested potatoes contain about 80% water and 20% dry matter and about 60–80% of the dry matter is starch. On a dry weight basis, the protein content of potato is similar to that of cereals and is very high in comparison with other roots and tubers. In addition, the potato is low in fat and it is also a good source of vitamins B1, B3, B6, folate, pantothenic acid, riboflavin and minerals, such as potassium, phosphorus and magnesium (FAO, 2008).

Apart from being a rich source of starch, potatoes contain good quantity of small molecules and secondary metabolites which can play an important role as bioactive molecules in the human diet (Friedman, 1997).

Changes in potato chemical composition mainly occur during storage and cooking (Burton, van Es and Hartmans, 1992; Liu, Tarn, Lynch and Skjodt, 2007) and vary depending on the cultivar and growing area (Abdel-Kader, 1990; Augustin *et al.*, 1978; Dwelle and Stallknecht, 1978). Each preparation method affects potato composition in a different way, due to leaching into cooking water and oil, destruction by heat treatment or chemical changes such as oxidation. Starch digestibility and the percentage of resistant starch of potatoes are affected by cultivars and cooking/cooling treatments (Mulinacci *et al*., 2008).

Among the different secondary metabolites, potatoes contain chlorogenic acid and its isomers that are involved in browning of the tuber after cutting or processing (Dao and Friedman, 1992) while flesh pigmented potatoes are a rich source of anthocyanins, in particular acylated derivates (Eichhorn and Winterhalter, 2005).

In fresh tubers the chlorogenic acid content varies from 100 to 190 mg/kg of fresh weight and contributes to its sensory properties (Work and Camire, 1996). For the most part, this compound and its analogues are localized in skin (nearly 50% of the

total content) and the percentage decreases gradually going inwards in the tuber (Friedman, 1997).

Chlorogenic acid is bioavailable in humans (Monteiro, Farah, Perrone, Trugo, Donangelo, 2007), may decrease the risk of type two diabetes (Legrand and Scheen, 2007) and slow the release of glucose into the bloodstream (Bassoli *et al.,* 2008), which could be relevant for lowering the glycemic index value of potatoes. Chlorogenic acid may reduce the risk of some cancers and heart disease (Granado-Serrano, Martin, Izquierdo-Pulido, Goya, Bravo and Ramos, 2007) and also shows anti-hypertensive effect (Yamaguchi *et al.,* 2008).

Anthocyanins are distributed in the skin and flesh of the tubers and their presence in plants plays a role of self-protection against biotic and abiotic stress and contributes to chemotaxonomic characterization (Ortega-Regules, Romero-Cascales, Lopez-Roca, Ros-Garcia, Gmez-Plaza, 2006); their content is strongly influenced by the growing area (Ieri, Innocenti, Andrenelli, Vecchio, Mulinacci, 2011). Red-fleshed potatoes have acylated glucosides of pelargonidin while purple potatoes have, in addition, acylated glucosides of malvidin, petunidin, peonidin and delphinidin (Brown, 2005a; Lachman and Hamouz, 2005). Their antioxidant and radical scavenging activities are well known *in vitro* and *in vivo* (Wang *et al.*, 1999; Tsuda, Katob, Osawa*,* 2000) and several studies in human subjects support the conclusion that consumption of anthocyanin-rich plants leads to an increase in serum antioxidant potential (Cao and Prior, 1998; Mazza, Kay, Cottrell, Holub, 2002). Anthocyanins are widely ingested by humans and their daily intake has been estimated at around 180 mg (Galvano *et al.*, 2004). The ORAC and FRAP assays revealed that the antioxidant levels in red or purple-fleshed potatoes were two or three times higher than in white or yellow-fleshed potatoes (Teow, Truong, McFeeters, Thompson, Pecota, Yencho, 2007), thus these varieties provide a natural source of anthocyanins which helps to reduce the risk of chronic diseases and age-related neuronal degeneration. However it has been reported that red and purple-flesh potatoes show higher content of cinnamic acids if compared to white or yellow tubers (Ieri *et al.,* 2011).

Recent studies pointed out that purple potatoes are hypotensive agents and lower the risk of heart disease and stroke in hypertensive subjects without weight gain (Vinson, Demkosky, Navarre, Smyda, 2012) and was hypothesized that polyphenols from pigmented potatoes would decrease oxidative stress and inflammation in humans (Kaspar *et al.,* 2011).

The present study aims at evaluating the anthocyanin and phenolic acid content in several cultivars of potato with yellow, red and purple flesh colour and at correlating these properties with the agronomic parameters. The effect of an industrial steam boiling process on the polyphenol content was evaluated studying changes in the concentration of chlorogenic acid, its isomers and total anthocyanins in fresh and cooked samples. Finally the antioxidant activity, using ABTS assay, was also estimated on the processed tubers.

2. Material and Methods

2.1 Materials

Six yellow- (Mz032, Mz046, Mz080, Primura, Melody, Universa) three violet- (Mz064, Vitelotte Noir, Mz128) and two pink/red-fleshed (Mz012, Mz011) varieties of potatoes were studied. The tubers were kindly provided by Pizzoli S.p.A. (Bologna-Italy), both fresh and after boiled steam cooking. For the three cultivar Primura, Melody and Universa the fresh tubers were not available. A summary of the morphological characteristics and of the common uses of all the cultivars is reported in Table 1.

Variety or code	Country of Origin	Maturity	Resistance to Disease	Skin Colour	Flesh Colour	Tuber Form	Yeld after Drying (% on FW)	Cooking Use
Mz011	Italy	Late	Not known	Pink	Pink	Long oval	16-19	Boiled, mashed
Mz064	Netherland	Medium late	Not known	Bown/ Violet	Blue/ Violet	Small long	20-23	Boiled, mashed and chips
Vitelotte Noir	France	Very late	-	Dark Violet	Dark Violet	Long Irregular	23	Boiled, mashed and chips
Mz128	Netherland	Late	Not known	Bown/ Violet	Light Violet	Round Oval	16-18	Boiled, mashed
Mz012	Netherland	Medium Late	Not known	DarkPink / Red	DarkPink/ Red	Long oval	17	Boiled, mashed
Mz032	United Kingdom	Medium early	Not known	Dark yellow and Purple	Dark Yellow	Small long	>23	Boiled, mashed
Mz046	United Kingdom	Medium early	Not known	Dark Yellow	Dark Yellow	Small long	>23	Boiled, mashed
Mz080	Netherland	Medium Late	Not known	Yellow with red eyes	Yellow	Oval	18-22	Multiuse
Primura	Netherland	Medium early	Many to fungi, viruses and insects	Yellow	Light Yellow	Oval	18-21	Multiuse
Melody	Netherland	Late	Many to fungi, viruses and insects	Yellow	Yellow	Round Oval	18-21	Multiuse
Universa	France	Medium early	Many to fungi, viruses and insects	Light Yellow	Light Yellow	Oval long	16-19	Boiled, salad

Table 1 Morphological characteristics of the analyzed cultivars. (–) unavailable data or not homogenous.

2.2 Industrial steam-boiling process in lab

The whole tuber with skin was submitted to a steam process as follow: cooking of potatoes was realized by boiling in a covered stainless steel pot on a moderate flame. NaCl (1%) and ascorbic acid was added during water boiling, and, when the salt was completely dissolved, potatoes (about 1 kg) were added to boiling tap water. The ratio potatoes/water was 1:4 p/v. At the end of cooking, samples were drained off for 30 s and cooled at room temperature.

The cooked tubers were then peeled and frozen as puree; these samples were used for the extraction of phenolic compounds.

2.3 Samples preparation

For each variety, the potatoes (1 g) were extracted at room temperature under stirring, twice with 30 mL of 70% EtOH adjusted to pH 2.0 by HCOOH, as already described in a previous study (Mulinacci *et al.*, 2008). The hydroalcoholic solutions were directly analyzed by HPLC/DAD/MS, according to our previous work (Ieri *et al.*, 2011) and used for the ABTS test.

2.4 HPLC/DAD/MS analysis

Analysis was carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP1100 MSD API-electrospray (Agilent-Technologies, Palo Alto, CA) operating in positive ionization mode under the following conditions: gas temperature 350 °C, nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 35 psi, quadrupole temperature 30 °C, capillary voltage 4000V, and applied fragmentors in the range 50-250 V.

The column was a Synergi Max RP 80 A (4 μm; 150 mm × 3 mm i.d.) from Phenomenex. The mobile phase was (A) water pH 2.0 acidified by orthophosphoric acid (only for HPLC/DAD) or formic acid (for the HPLC/DAD/MS analysis) and (B) acetonitrile. The following multistep linear gradient was applied: from 95% to 78% of A in 8 min, 4 min to reach 74% A, then 13 min to arrive at 65% A, and finally 3 min to reach 100% B with a final plateau of 4 min. Total time of analysis was 32 min, flow rate was 0.4 mL/min, and oven temperature was 26 ± 0.5 °C as already described in our previous study (Ieri *et al.*, 2011).

2.5 Quantitative determination of phenolic acids and anthocyanins

The phenolic acids were evaluated by HPLC/DAD using a six-point calibration curve of chlorogenic acid (Extrasynthese-Genay Cedex-France) at 330 nm (r^2 0.999), while the anthocyanin content was determined by HPLC/DAD using a five-point calibration curve of malvin chloride (mw 691 from Extrasynthese-Genay Cedex-France) at 520 nm (r^2 0.999).

2.6 Antioxidant activity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) method

The ABTS method described by Lachman *et al.* (2012) was used for the antioxidant activity determination.

The hydroalcoholic extracts were diluted with distilled water (100 times) and immediately subjected to reaction with the radicals; absorbance was measured after the addition of a 200 μL sample to 2 mL of radical solution after 2 min. For the blank experiment 200 μL water were added. The antioxidant activity was calculated as follow by the equation:

% inibition ABTS = $[(A_0 – A_t)\ A_0] \times 100$

In agreement with Lachman *et al.* (2012), 54.8 mg ABTS (Sigma-Aldrich) were dissolved in 20 mL phosphate buffer (pH 7.0; 5mM) and activated to $ABTS^{\cdot+}$ radical adding 1g of MnO_2 (activation time 20 min). Then the solution was centrifuged, filtered and diluted with the buffer solution to obtain $A_{734}(t_0) = 0.800 \pm 0.02$ nm. Absorbance of the solution was measured at a wavelength of 734 nm. Values are the means of three replicates.

3. **Results and Discussion**

3.1 Phenolic acid and anthocyanin contents

Phenolic acids and anthocyanins have been analyzed using HPLC methods and the structures were confirmed by their UV-Vis and mass spectra. Firstly a chemical characterization in terms of phenolic content applying the extraction method

previously described (Ieri *et al.,* 2011) was carried out on the fresh tubers. The same extraction was applied on the potatoes after steam cooking showing that the HPLC/DAD profiles after processing were the same as those obtained for the respective fresh tubers (Figure 1 and Figure 2). The same phenolic acid pattern was highlighted and the main phenolic acids detected were 3-caffeoylquinic, 5-caffeoylquinic, 4-caffeoylquinic and ferulic acids for all of the considered samples, with the chlorogenic acid as main component (Figure 1).

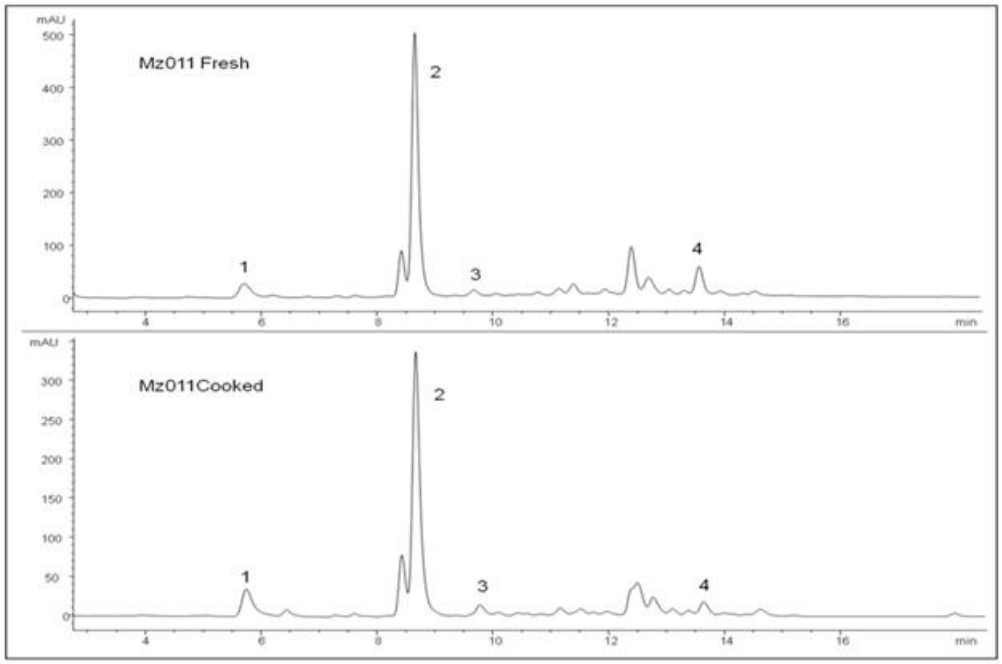

Figure 1. HPLC chromatogram at 330 nm showing the effect of boiling treatment on phenolic acids in Mz011 cultivar. 1) 3-caffeoylquinic acid 2) 5-caffeoylquinic acid or chlorogenic acid 3) 4-caffeoylquinic acid 4) ferulic acid.

The anthocyanin patterns of red- and purple-fleshed cultivars resulted more complex as shown in Figure 2, that reports the HPLC profiles at 520 nm showing the anthocyanins in Mz011 cultivar, chosen as an example, before and after the cooking treatment. The anthocyanins identified were acylated glycosides of pelargonidin, malvidin, petunidin, peonidin and delphinidin and specifically, the

rutinosides in C3, acylated with a cynnamoil residue in C4 of the rhamnose unit, were the dominant forms.

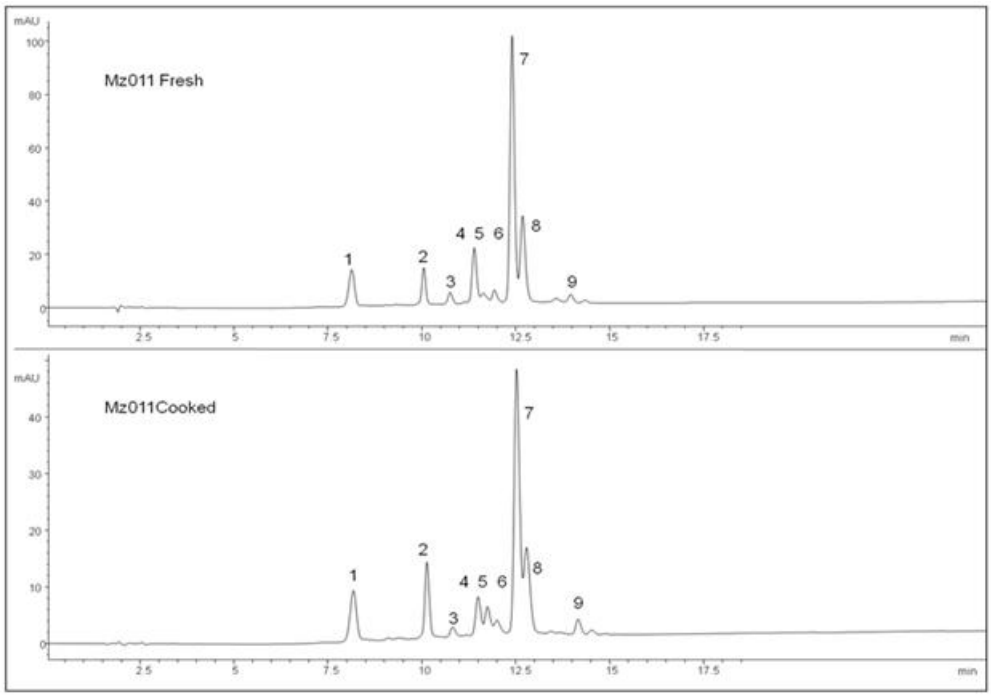

Figure 2. HPLC chromatogram at 520 nm showing the effect of boiling treatment on anthocyanins in Mz011 cultivar. 1) pel 3-O-rut-5-O-glu 2) pel 3-O-rut 3) pel derivative 4) pel 3-O-caf-rut-5-O-glu 5) pel 3-O-cis-p-coum-rut-5-O-glu 6) pel derivative 7) pel 3-O-p-coum-rut-5-O-glu 8) pel 3-O-ferul rut-5-O-glu 9) pel 3-O-p-coum-rut.

The anthocyanin profile was determined for all five red and purple pigmented potatoes and the identification of the molecules was carried out by their UV-Vis and mass spectra in positive ionization mode at 120 fragmentation energy and by comparison with literature data (Mulinacci *et al.,* 2008).

From the quantitative point of view, significant variations were pointed out in the concentration of the phenolic compounds in the steam boiled tubers.

As summarized in Figure 3a, the content of total phenolic acids on average was determined for the eight coloured varieties and ranged from 38.14 to 1153.02 μg/g of fresh material (FM) in fresh potatoes and from 114.32 to 1352.1 μg/g FM in cooked potatoes. The highest total phenolic acid content was found in Vitelotte

Noir cv (1153.02 μg/g FM) for the fresh samples and Mz012 cv (1352.1 μg/g FM) for the cooked samples.

With regard to the yellow-fleshed cultivars, average total phenolic acid content values ranged from 38.14 to 99.4 μg/g of fresh material (FM), with changes in their content less evident after cooking (from 114.32 to 132.41 μg/g FM). However the total amount of these metabolites in the yellow-flesh variety is notably lower in comparison to the content observed in the pigmented cultivars according to our previous works (Mulinacci *et al.* 2008; Ieri *et al*., 2011). A recent study reported a concentration of phenolic acids in purple- and red-fleshed cultivars from three to four times higher with respect to white fleshed cultivars (Ezekiel, Singh, Sharma, Kaur, 2013).

According to these data, three other yellow-flesh potatoes (Primura, Melody and Universa) provided by Pizzoli S.p.A. and analyzed after steam cooking, showed a total phenolic acid content ranged from 52.66 to 111.36 μg/g, values in agreement with those of the other processed yellow-fleshed cultivars.

On the opposite of what commonly expected, after cooking in all the yellow-flesh varieties the content of phenolic acids increased in relation to the cultivar, the highest difference between fresh and cooked tubers was for the cultivar Mz080. As shown in Figure 3a, a significant increase of total amount of phenolic acids was also found in three cooked red and purple pigmented potatoes in comparison with uncooked tubers (Mz064, Vitelotte Noir and Mz012). The highest increase (2.5 times) was for the cultivar Mz012, curiously characterized by the lower total phenolic acid content in the fresh sample. These results agree with literature data that showed greater levels of phenolic acids in cooked potatoes than in uncooked ones (Mulinacci *et al.* 2008; Blessington, Nzaramba, Scheuring, Hale, Reddivari and Miller, 2010, Burgos *et al.* 2013). This phenomenon can be explained because cooking could produce hydrolysis of different components releasing phenolic compounds and making them more available for extraction (Burgos *et al*., 2013). Similarly Lachman *et al.* (2012) found that total phenolics, chlorogenic acids, flavonols and vitamin C did no significantly decrease after cooking by microwaving, baking, boiling, steaming or stir-frying methods and this increase

could be attributed to disruption of plant cell walls providing better extractability, breaking of chemical bonds of higher molecular weight polyphenols and forming soluble low molecular weight polyphenols and their interconversion.

Again, to confirm that the thermal changes are dependent on cultivar, it can be pointed out that the total phenolic acid concentrations of the Mz011 and Mz128 cultivars determined in boiled tubers were lower than in raw sample, in agreement to other studies (Takenaca, Nanayama, Isobe, Murata, 2006; Perla, Holm, Jayanty, 2012). This specific behaviour may be related to concomitant factors: browning phenomenon during the cut, reduced pulp surface exposed to hot water, percentage of water absorbed by the starch during cooking and mainly, the presence of peel (Mulinacci *et al.*, 2008). It is well known that potato peel contains high quantities of phenolics. Since 1989 Mondy and Gosselin found that the potatoes cooked with peel had a greater amount of total phenols in the cortex and internal tissues and this has been attributed to the migration of phenolics from the peel into both the cortex and internal tissues of the tuber (Ezekiel *et al.*, 2013).

Overall, after this industrial cooking the anthocyanin content decreased in a cultivar-dependent mode, as reported in Figure 3b, and in particular Mz128 and Mz064 showed the most significant loss (67% and 36.7% respectively) of these compounds. The findings of our study highlighted the highest anthocyanin amount for the cultivar Mz064, ranging from 0.7 mg/g for the uncooked, to 0.4 mg/g for the cooked tubers (Figure 3b). Only in one sample (Mz012) was evidenced a significant increment for the anthocyanin content. These data are not new, in fact recent paper (Lachman *et al.,* 2012, Brown, Durst, Worlstad, De Jong, 2008) pointed out similar results for the anthocyanins in red- and violet- fleshed cooked potatoes. Cooking methods and cultivars are, with respect to total anthocyanins, significant sources of variation. Lachman *et al.* (2012) observed that baking, microwaving or steaming were the most efficient processing methods in preserving of anthocyanins when compared to boiling. Brown *et al.* (2008) found that microwaving and boiling preserved total anthocyanins more than frying or baking. Anyway, in all these cooking treatments the increase in total anthocyanin content was always cultivar dependent (Lachman *et al.,* 2013).

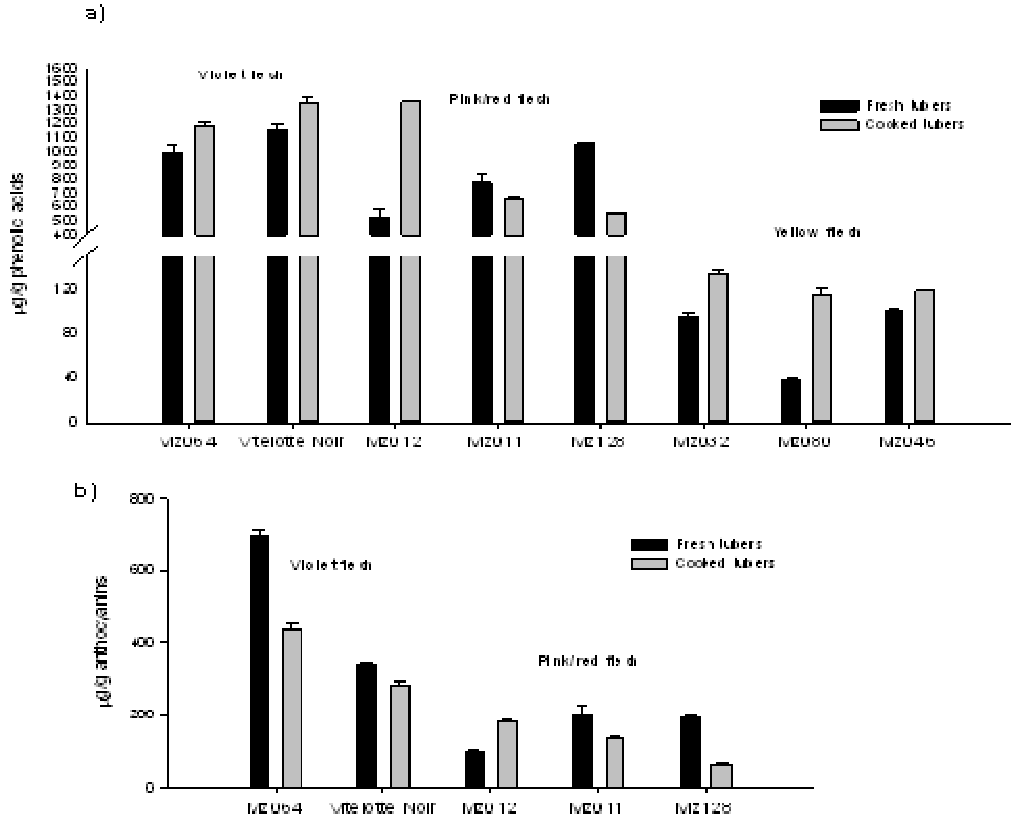

Figure 3. a) Total phenolic acids in fresh and cooked red-, purple- and yellow-flesh cultivars of *Solanum tuberosum* L. b) Total anthocyanins in fresh and cooked pigmented varieties of *Solanum tuberosum* L.

Overall the increase in the concentration of phenols after cooking was attributed to an increase in recoverable compounds, as there is little biosynthesis of these molecules during thermal treatments. Cooking may facilitate the extractability of these compounds by altering the matrix, resulting in higher recoveries and inactivating enzymes that otherwise oxidize these compounds (Navarre, Shakya, Holden, Kumar, 2010).

3.2 Antioxidant activity

As demonstrated by many authors (Reyes, Miller, Cisneros-Zevallos, 2005; Hamouz *et al.,* 2011, Ezekiel *et al.,* 2013), potatoes can be significant source of

natural antioxidant in diet and have several secondary constituents which contributes to the physiological defence against oxidative and free-radical-mediated reactions.

Considering that potatoes are usually served after cooking, the antioxidant activity has been evaluated only for cooked tubers. As described in Figure 4, considerable difference in antioxidant activity between these cultivars were observed, mainly in agreement with differences of phenolic content between yellow-, red- and violet-fleshed potatoes.

As expected, the lowest antioxidant activity was achieved by the group of yellow flesh variety (Figure 4), averaging 0.36 μg/mL ascorbic acid, instead in the group of red- and violet-fleshed cultivars (Figure 5) this value was more than 3 times higher considering the variety with the highest total anthocyanin content (Mz064).

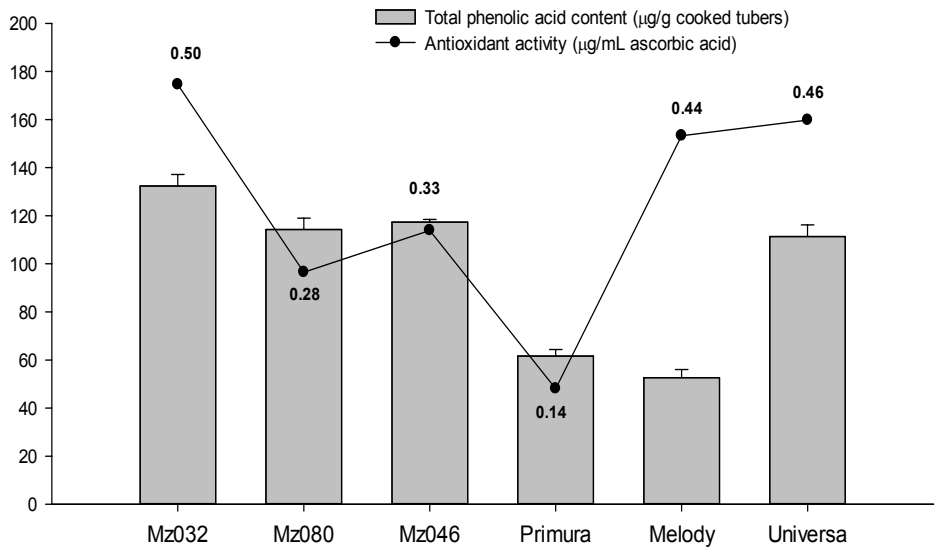

Figure 4. Comparison of total phenolic acid content and antioxidant activity of yellow-fleshed potatoes.

Among the yellow-fleshed cultivars, the sample Mz032 presented the highest activity (0.50 μg/mL ascorbic acid) according to its highest content in total phenolic acids (132.4 μg/g of cooked puree), as reported in Figure 4. For the yellow-fleshed

cultivars a positive correlation was found between their activity and the content of phenolic acids, except for cv. Melody.

More differences in antioxidant activity were found for red and violet-fleshed potatoes (Figure 5). The analysis showed again a strong correlation between ascorbic acid equivalents and total anthocyanin content suggesting that these compounds are mostly responsible for the antioxidant properties, according also to previous results (Lachman *et al.,* 2009; Lachman *et al.,* 2012). Otherwise not a similar correlation was observed for these cultivars with their total phenolic acid content (Figure 6).

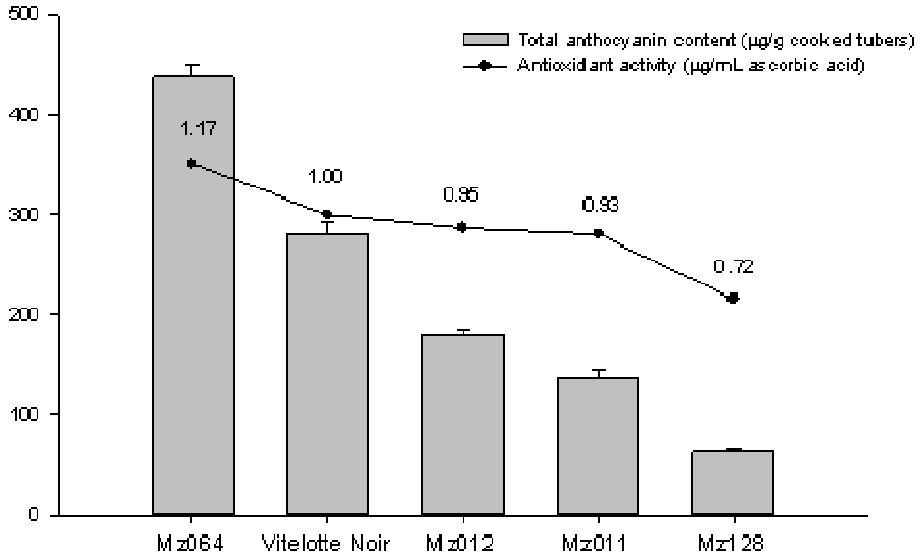

Figure 5 Comparison of total anthocyanin content and antioxidant activity of red and purple-fleshed cultivars.

Reddivari *et al.* (2007) estimated that chlorogenic acid contributed approximately from 28 to 45% of antioxidant activity. As reported in Figure 3a, the pigmented genotypes with red and violet flesh contained considerably higher levels of chlorogenic acid isomers than non-pigmented genotypes but our results did not show a correlation between antioxidant activity and total phenolic acid content in these cultivars (Figure 6). So it can be concluded that an high total phenolic acid

concentration, mainly chlorogenic acid and its isomers, does not induce necessarily higher antioxidant activity values in red and violet potatoes. On the opposite, the positive correlation found for the total anthocyanin content can be explained with the highest antioxidant power of these pigments with respect to the cinnamoyl derivatives (Figure 5).

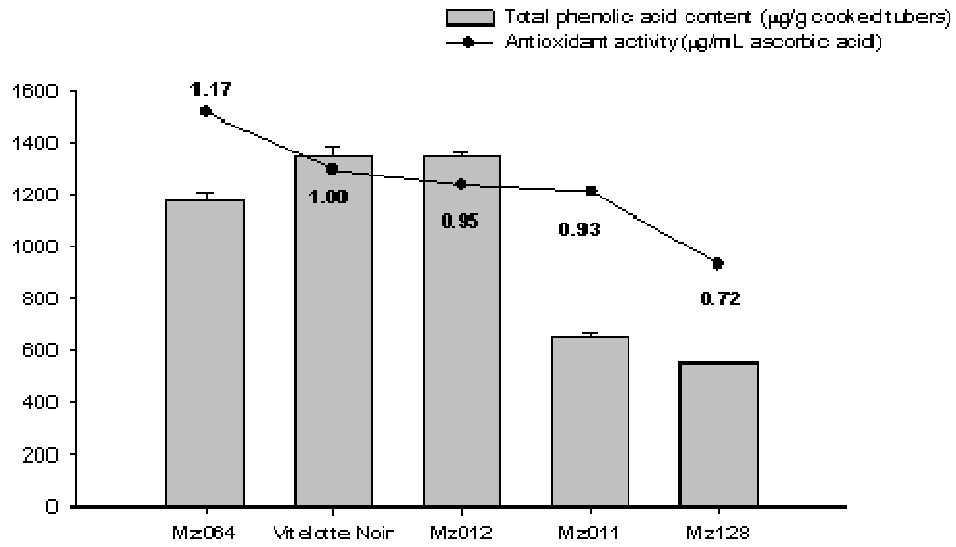

Figure 6 Comparison of total phenolic acid content and antioxidant activity of red and purple-fleshed cultivars.

In agreement to our data are also the results obtained by Brown (2005a), Brown *et al.,* (2005b) and Lachman *et al.* (2009), that showed higher antioxidant activity mainly caused by anthocyanins in the cultivars with pigmented flesh, in which the antioxindant power significantly exceed that of traditional yellow-fleshed varieties (Hamouz *et al.*, 2011; Brown *et al.,* 2008).

4. Conclusions

These data highlight the possibility to use the pigmented potatoes to increment the daily intake of the anthocyanins, an important class of bioactive molecules, because only a partial decrement is obtained after a typical industrial cooking process.

As expected the red and violet- fleshed potatoes show the highest efficacy as radical scavenger in the ABTS test. This test is suitable to clearly distinguish between pigmented and yellow tubers and within each group the antioxidant activity is well related to the total phenolic content.

Whereas in potatoes with white or yellow flesh colour prevalent contributor to antioxidant activity is chlorogenic acid and in violet and red-fleshed potatoes the major contributors to antioxidant activity are indubitably the anthocyanins.

Our results indicate that red and violet-fleshed potatoes show a significantly higher antioxidant activity than the yellow-fleshed cultivars and that antioxidant activity and total anthocyanin content are high-correlated. Using violet-fleshed potatoes in the cuisine would help to support the daily intake of these acylated anthocyanins and consequently, these cultivars could be a promising source of strong antioxidants in human nutrition.

In conclusion, highest-anthocyanin coloured-flesh potatoes cultivars can be suitable for specific processing and could represent an high-phytonutrient source for certain antioxidants not so frequently present in other foods, in addition to offer an alternative to classic yellow-flesh potatoes in human nutrition.

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An innovative extraction method for phenolic compounds and volatile terpenes from the same leaf sample of *Rosmarinus officinalis* L.

Talanta, Submitted

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Abstract

In this work the possibility to optimize a unique procedure to extract, in successive steps, the volatile terpenes and the phenolic compounds from the same sample, working on fresh rosemary leaves, has been explored.
A significant increment of the extraction yields has been observed applying this innovative method with respect to traditional processes.
The results highlighted the suitability of this innovative extractive procedure to treat aromatic plants, providing a useful tool to obtain better yields in volatile terpenes and new extracts for the botanical market.

Keywords: ethanol extraction, phenols, terpenes, pentane and hexane extraction, vitamin C

Introduction

Mediterranean basin is endowed with a rich wealth of aromatic plants such as rosemary, *Rosmarinus officinalis* L. (Labiatae), a small evergreen shrub whose health benefits of its essential oil and properties for preservation of food are recognized since ancient times. To date it is known that the rosemary leaves contain both volatile components that characterize the essential oil, and antioxidant phenolic compounds belonging to different chemical classes and showing a different polarity and solubility.

Rosemary essential oil contains mainly monoterpenes and monoterpene derivatives (95–98%), the remainder (2–5%) being sesquiterpenes (Angioni *et al.,* 2004). Volatile mono- and sesquiterpenes are main constituents of the essential oil used as flavors and fragrances down through history in the ancient civilization of Egypt, India, Greece and Rome. Nowaday, rosemary extracts are used not only to obtain essential oils but also to prepare phenolic extracts that are increasingly employed to provide natural alternatives to synthetic antioxidant and artificial preservative additives in foodstuffs (The EFSA Journal, 2008; Moreno *et al.,* 2006) or as component of cosmetic (Lee *et al.,* 2011). The antioxidative property and part of the antimicrobial activity of the fresh and dried leaves of rosemary are related to the non-volatile phenolic compounds such as carnosic acid, carnosol, rosmarinic acid and a group of minor flavonoids. As reported in the ESCOP monograph and more recently highlighted in a dictionary of phytotheraphy (Campanini, 2004), the rosemary leaf extracts are commonly used also in the traditional medicine against several diseses as dyspepsia, dysmenorrhea and rheumatic diseases.

Among the numerous activities of the phenolic extracts from rosemary it can be highlighted the hepatoprotective (Sotelo-Felix *et al.,* 2002; Gutiérrez *et al.,* 2009), antihyperglycemic (Al-Hader *et al.,* 1994; Bakirel *et al.* 2008), antiulcerogenic (Dias *et al.,* 2000) and antibacterial properties (Celiktas *et al.,* 2007).

Basing on our knowledge, a chemical characterization in terms of volatile and phenolic compounds using the same sample of rosemary leaves has not yet been evaluated.

Being useful for integrated studies on numerous samples to use a suitable method to extract both the volatile and phenolic components from the same foliar tissue, this possibility was explored. The aims were to optimize a unique procedure to extract, in successive steps, the volatile terpenes and the phenolic compounds from the same sample working on fresh rosemary leaves. Specific GC/MS and HPLC/DAD/MS methods for the volatile terpenes and to determine the antioxidant phenolic fraction were applied. The results highlighted the suitability of this extractive procedure to treat aromatic plants, providing a useful tool to obtain better yields in volatile terpenes and new extracts for the botanical market.

Materials and methods

Plant material

Mature leaves were collected on May 2011, from adult plants of a single clone of *Rosmarinus officinalis* L. growing in the plant nursery situated at the National Research Council in Firenze. The dried samples (EtC 20m) were obtained after a freeze-drying procedure.

Traditional (conventional) extractive method (TEM)

As shown in Figure 1, rosemary samples were frozen in liquid nitrogen and ground into a porous ceramic mortar (grinding time about 1 min). The grinding is made to allow the breakdown of cellular structures containing terpenic constituents, avoiding the loss of these substances, which are extremely volatile.

For each sample, 0.5 g of ground material were extracted with 3 mL of *n*-pentane or 3 mL of *n*-hexane, using tridecane as internal standard. The extraction process was performed for 24 hours in shaker at 1000 rpm at 24 °C. The *n*-pentane or *n*-hexane extracts were filtered through 0.45 μm filters, stored in vials at -20 °C before GC/FID analysis (Figure 1, Pen or Hex samples). The solid residue was recovered and subjected to further extraction, according to the procedure previously described (Mulinacci *et al.*, 2011), for the recovery of the phenolic compounds. In particular,

leaves were extracted with ethanol (two steps) by the alternation of magnetic stirring and sonication in ultrasounds. The ethanolic extract obtained after filtration was submitted to a liquid/liquid extraction with pentane or hexane (1:1, v/v) to remove part of the chlorophylls. The residual ethanol solution was directly analyzed by HPLC (Figure 1, EtC sample).

To prevent the phenolic oxidation, different amounts of vitamin C (5-20% w/w) were added to some samples before the grinding (Figure 1, EtC+VitC samples).

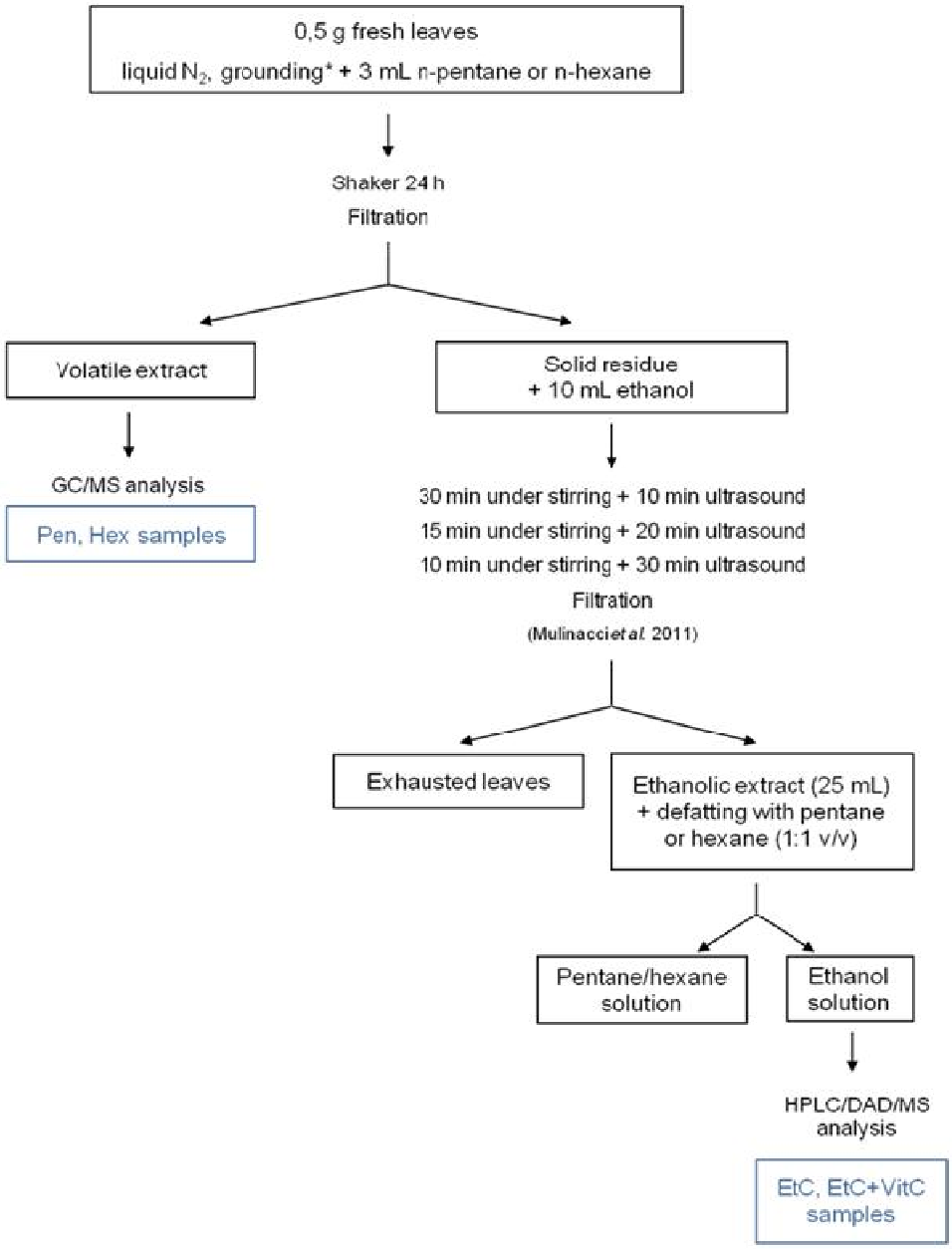

Figure 1. Traditional extractive method (TEM). *different amounts of vitamine C (5-20% p/p) were added to some of the samples

Innovative extractive method (IEM)

The volatile terpenes were extracted as second step after the ethanol extraction of phenolic compounds (Figure 2). Appliyng this method, 0.5 g of ground material were extracted with 10 mL of ethanol according to Mulinacci *et al.* (2011). The ethanolic extract obtained after filtration was submitted to a liquid/liquid extraction with pentane or hexane (1:1, v/v); the ethanol extract was analyzed by HPLC (Figure 2, Et-REF) and the *n*-pentane/*n*-hexane solutions were analyzed by GC/MS (Figure 2; Def-Pen, Def-Hex). At the same time, the solid residue was recovered and subjected to the extraction of volatile terpenes. The resulting pentane/hexane solutions were analyzed by GC/MS (Figure 2; Et-Pen, Et-Hex samples).

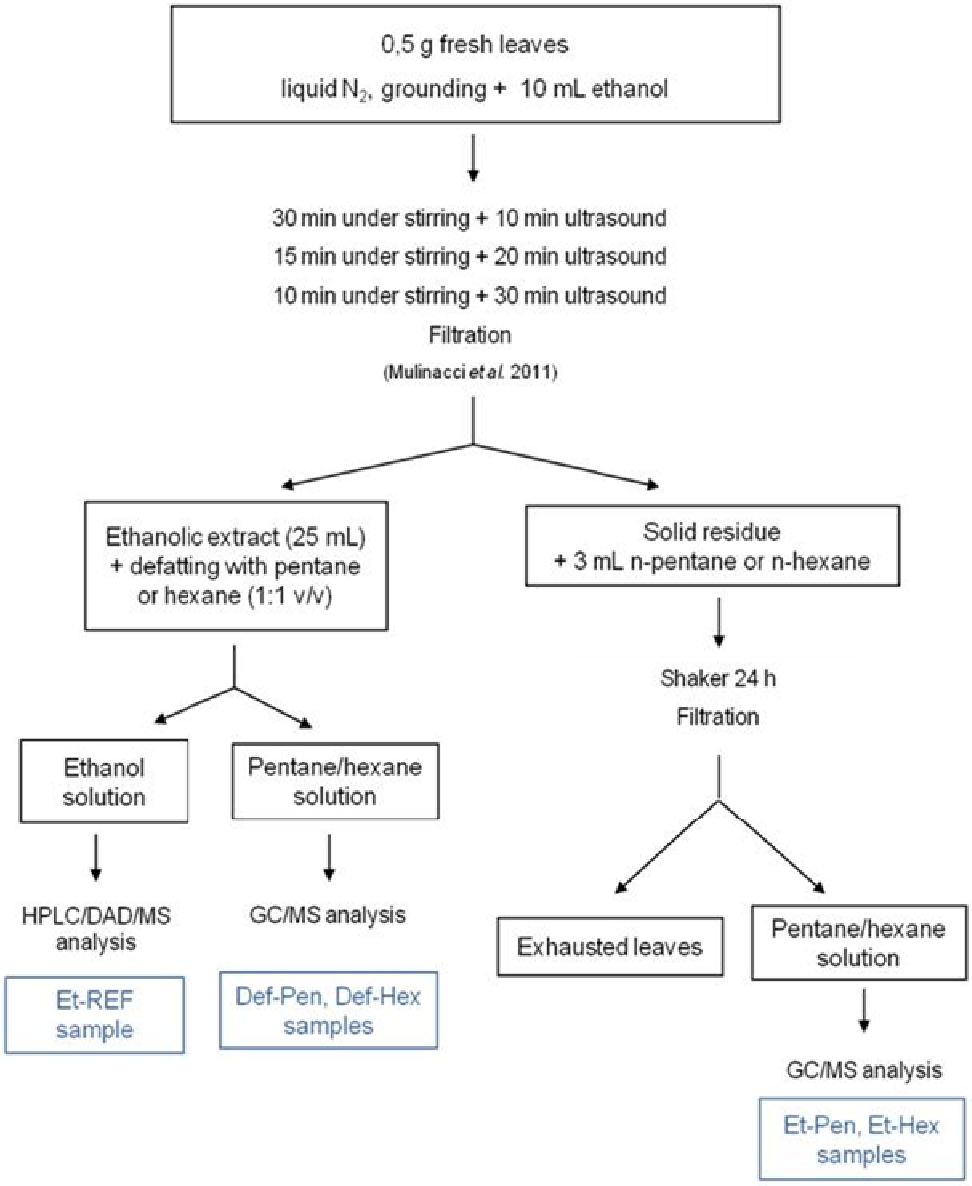

Figure 2. Innovative extractive method (IEM)

GC/FID and GC/MS analyses

The analyses were performed on a GC/MS system from Perkin-Elmer Technologies composed of a Gas Chromatograph Perkin-Elmer AutoSystem XL equipped with an automatic sampler for liquid sample injections coupled to a TurboMass mass spectrometer.

To obtain the separation of the enantiomeric monoterpenes a Elite-Betacydex Betacyclodextrin capillary column 30-m-long and 0.25-mm-diameter supplied by Perkin-Elmer was used. GC analysis was carried out using hydrogen as carrier gas at 2.0 mL min^{-1} by a flame ionization detector at 250 °C and at injector temperature 230 °C. The oven temperature programming started at 40 °C for 3 min and increased to 200 °C, at 1 °C min^{-1}; the final temperature of 200 °C was maintained for 10 min.

The mass spectrometer was operating with a electron ionisation of 70 eV, scanning the mass range from 35 to 350 m/z. Ion source temperature was 200 °C. The GC/MS control and data elaboration were performed by a Perkin-Elmer Technologies TurboMass 5.4.2.1617 ver. Chemstation software. The mass spectrometer was calibrated using perfluorotributhylamine, as the calibration standard, with the Chemstation software. High-purity components were obtained from Fluka, Aldrich and Acros.

The analysis of pure standards in the same analytical condition allowed the attribution of some GC/MS signals while, when a pure standard was not available, the identification was attempted comparing the recorded mass spectra with a MS spectral database (Wiley library) and taking in account the chromatographic order of elution.

HPLC/DAD/ESI/MS analyses

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (all from Agilent Technologies, Palo Alto, CA, USA).

A 150 mm × 2 mm i.d., 4 μm Fusion, RP18 column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN; the multi-step linear solvent gradient used was: 0–15 min 15–25% B; 15–25 min, 25–35% B; 25–35 min 35–50% B; 35–40 min 50–100% B with a final plateau of 8 min at 100% B; equilibration time 10 min; flow rate 0.2 mL min^{-1} and oven temperature 26°C; injection volume 5 μL. The analysis conditions were the same described in a previous study (Mulinacci *et al.*, 2011).

Quantitative evaluation

Relative amount (proportion of profile) of each monoterpene was expressed as a percentage of total monoterpenes, while each sesquiterpene was calculated as a percentage of total monoterpenes plus sesquiterpenes.

Absolute amounts of volatile terpenes were determined by comparison with the tridecane internal standard, and expressed as mg/g fresh weight (FW).

The quantitative evaluation of the main phenolic constituents was performed through the use of two external standards, rosmarinic acid at 330 nm and carnosic acid at 284 nm. According to a previous work (Mulinacci *et al.*, 2011), rosmarinic acid was used to quantify also all the flavonoids while carnosic acid to determine the non volatile diterpenoids (carnosic acid and derivatives). The calibration curve of rosmarinic acid (Sigma-Aldrich) was in a linearity range between 0.1 μg and 9.4 μg with a r^2 0.9999; the calibration curve of carnosic acid (Sigma-Aldrich) was in the linearity range of 0.05-3.4 μg with r^2 0.9998.

Statistical analyses

Data were not normally distributed (Kolmogorov–Smirnov one sample test) and were analyzed by the non-parametric Kruskal–Wallis ANOVA followed by the Mann–Whitney U-test for multiple comparisons using SYSTAT 12.0 software (Systat Software Inc., Richmond, California, USA). Differences were accepted when significant at the 5% level.

Results and discussion

Our goal is to use a unique procedure to sequentially extract the volatile terpenes and the phenolic compounds from a single sample, in order to get a more complete biological picture of the same foliar tissue in response to different biotic and abiotic stress conditions. Moreover, this extractive method can be an useful tool analyzing numerous samples, particularly when available in small amount. Within this work this analytical approach has been evaluated working on fresh leaves of rosemary.

A traditional approach (TEM) was firstly applied to recover the volatile fraction and, subsequently, the antioxidant phenolic compounds. A method suitable to well characterize and quantify all the phenolic constituents of the leaves (Mulinacci *et al*., 2011) was used to evaluate the percentage of recovery of these compounds applying the TEM and to obtain the reference samples (Figure 2, Et-REF).

Through the TEM, it was pointed out that a pretreatment of rosemary leaves with lipophilic solvents such as pentane or hexane from 1 to 24 hours of contact, induced a strongly decrease of the major phenols, mainly rosmarinic acid (Figure 3a, EtC sample). Furthermore, by the HPLC/DAD analyse of this extract it was observed a considerable decrement of carnosic acid with a corresponding increment of its typical oxidized products, carnosol and rosmanol.

These results were highlighted already after only 20 minutes of contact of the fresh leaves with these solvents (Figure 3a, EtC 20m sample). In addition, the fast degradation of the phenolic components after the preliminary extraction of the volatile compounds, was clearly confirmed by foliar browning indicating the formation of oxidized compounds frequently produced in the damaged vegetables. Consequently, an enzymatic reaction involving endogenous phenol oxidases was hypothesized. Moreover by adding antioxidant (e.g. BHT) and by removing the oxygen during the extraction procedure (data not shown), the chemical oxidation was excluded. Nevertheless it was unclear how the enzymatic activity was possible in presence of lipophilic organic solvents and, to explain this phenomenon, it was considered the possible role of the native water in the fresh leaves. The presence of water during the extraction of the volatile compounds allowed to obtain a two phase

system, in which the aqueous phase, even if in small amount, was enough to allow the activity of the oxidative enzymes and to promote the carnosic acid conversion into carnosol, as previously demonstrated (Masuda *et al*., 2002).

To confirm this hypothesis an extraction on dried rosemary leaves was carried out (Figure 3b). The results evaluated by the chromatographic analyses confirmed the crucial role of the water since no reduction of the rosmarinic acid content was observed; nevertheless, a loss in the total terpenoid and flavonoid contents was showed one more time. These data confirmed that enzymatic oxidation of rosmarinic acid in the fresh leaves, not observed in the correspondent dried samples, was rapidly obtained in presence of the native water (about 50-60% of the fresh weight) that provided a microenvironment where the enzyme can act, despite the presence of pentane or hexane. The next tests were aimed to inhibit the phenol oxidases by using strong inorganic acids (HCl and H_2SO_4 0.1 N as final concentration) added immediately after grinding during TEM. Even though these acidic conditions, the enzymatic oxidation of rosmarinic acid was almost complete within a short time, suggesting a rapid kinetics of degradation of these enzymes.

These results pointed out the complexity to operate on the same fresh foliar tissue of rosemary for extracting sequentially the volatile and the phenolic components, without a significant loss in rosmarinic acid and terpenoid content. At the same time it has been pointed out, for the first time, that the endogenous phenol oxidases in rosemary maintain their activity also at low pH values, demonstrating a strong resistance at these adverse conditions.

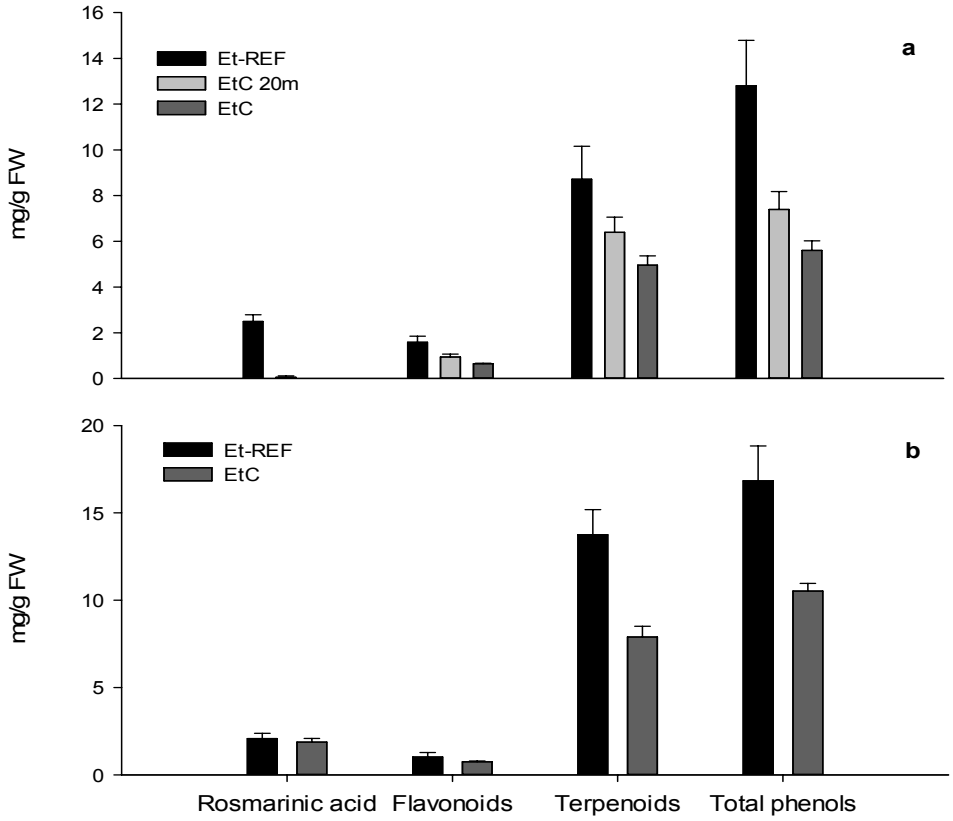

Figure 3. Comparison in terms of phenolic content among different extracts (Et-REF, EtC, EtC20m) obtained applying the TEM. The extraction was applied on fresh (a) and on freeze-dried (b) leaves

The final test that we carried out to prevent the phenolic oxidation applying TEM has been to add different amounts of vitamin C (5-20% w/w) to rosemary leaves before the grinding. Vitamin C has been chosen because of its solubility in water, in which commonly acts as strong antioxidant.

The results obtained by GC/MS analysis concerning the volatile fraction showed that the addition of vitamin C did not cause significant differences in the concentration of volatile terpenes, compared to the data obtained by the conventional extraction with pentane or hexane (Figure 1, Pen and Hex samples). Regarding the phenolic compounds, the vitamin C showed only a partial protective

action against rosmarinic acid oxidation (Figure 4) even at very high doses (20% w/w).

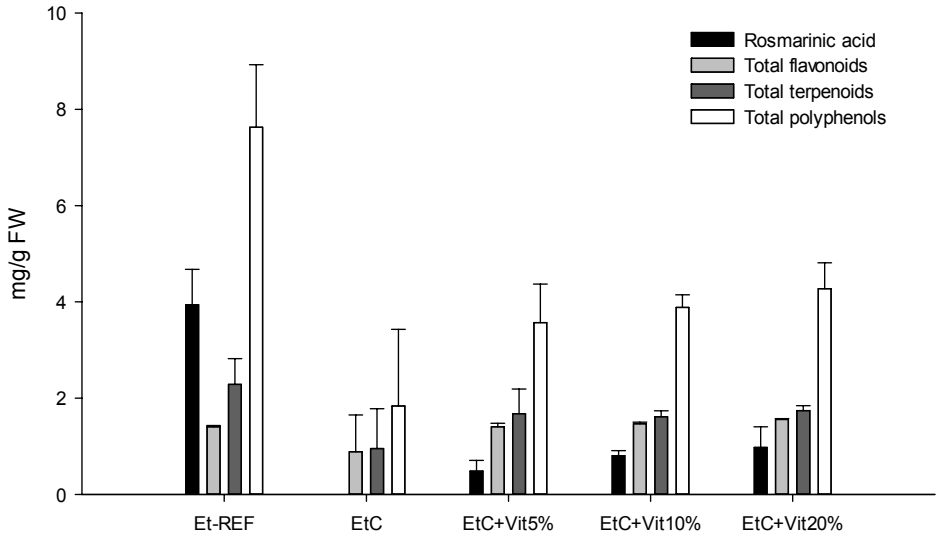

Figure 4. Effect of vitamin C on phenolic content in the extracts obtained according to Figure 1

In light of these results, the conventional extractive approach (TEM) has been reversed and the fresh leaves samples were firstly extracted with ethanol to recover the phenolic compounds and then using pentane or hexane (IEM) (Figure 2).

A significant increment of the extraction yields has been observed applying this innovative method (Figure 5, Et-Pen, Et-Hex extracts) with respect to the traditional approach (TEM) (Figure 5, Pen and Hex samples). This result was particularly interesting considering that the higher extractive yields were obtained despite a small amount of volatile terpenes were removed by the ethanolic extraction (Figure 2 and Figure 5; Def-Pen, Def-Hex samples). An explanation could be that the pretreatment with ethanol, associated to a physical treatment with ultrasound for about 2 hours, helped to break the cellular structures promoting the following penetration of the lipophilic solvent in the leaf tissue (Toma *et al.*, 2001; Chemat *et al*., 2011).

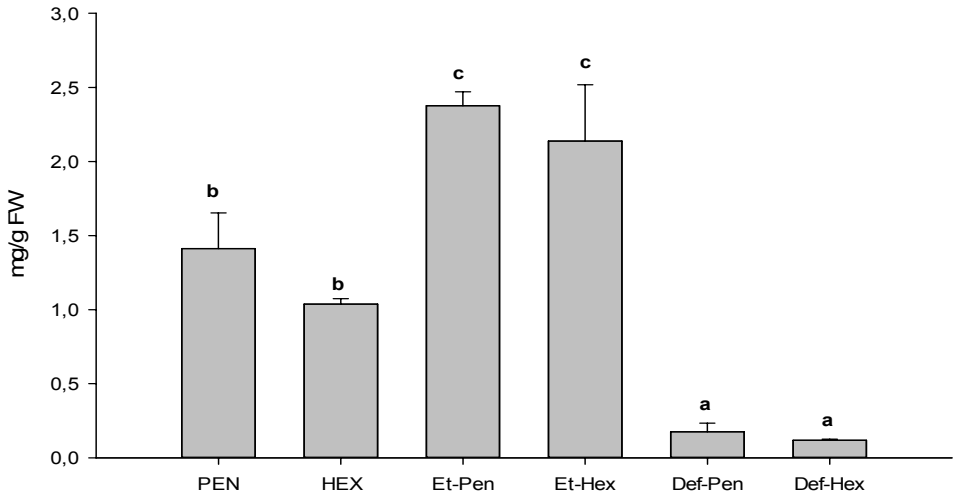

Figure 5. Comparison of the total terpene content in the extracts obtained by TEM and IEM

Moreover the qualitative composition of the terpenic extracts obtained with this two different extractive approaches (TEM and IEM) was evaluated to verify if the increased yields were associated to a similar or different GC profile. In Figure 6 was reported the amount of the main volatile terpenes in the extracts obtained using the two different methods (TEM and IEM).

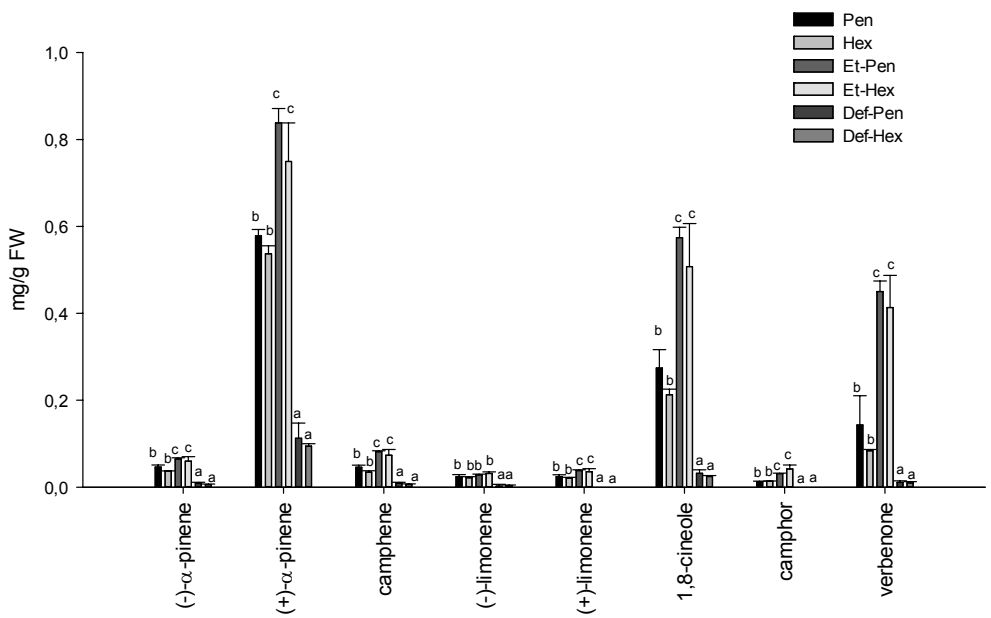

Figure 6. Content of the main terpenes in the extracts obtained by TEM and IEM

The concentration of the individual compounds reflected the trend observed for the total terpenoid content (Figure 5). All the samples treated with pentane (Figure 6; Pen, Et-Pen and Def-Pen) have provided higher yields than the samples extracted with hexane; pentane, because of its greater extractive capacity, proved to be the most suitable solvent for the extraction of the volatile fraction. The compounds (+)-α-pinene, 1,8-cineole and verbenone have proved to be the most aboundant in the analyzed extracts.

Conclusions

The conventional method (TEM) has not been able to guarantee a complete extraction of the phenolic compounds despite the use of potent antioxidants such as vitamin C.

On the other hand, the innovative approach proposed (IEM) ensured the recovery of all the phenolic components and also a consistent increase in extraction yields of the volatile fraction.

The findings of this study can be taken into account in planning the processing on rosemary leaves, or on similar aromatic plants, to avoid anomalous and unexpected results during the sample treatment.

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Phenolic extracts from rosemary leaves: an *in vitro* evaluation of their antibacterial activity

Natural Product Communications, Submitted

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Abstract

The aim of this research was to evaluate and compare the antimicrobial action of three phenolic extracts from rosemary leaves against two Gram-negative bacteria, *Escherichia coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 15442), and two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 82221). These species are usually required to evaluate the bactericidal activity of common chemical disinfectants. By the preliminary results obtained by disk diffusion method, Gram-positive bacteria were more sensitive to the extracts than Gram-negative ones and *S. epidermidis* showed higher inhibition zones (13.1 mm to 18.6 mm). The results by broth microdilution assay indicated that *E. coli* was the more susceptible strain against all the extracts (MBC $\leq$ 70 μg/mL), in contrast to the data obtained by disk diffusion method. The sample containing the highest concentration of flavonoids but the lower content of terpenoids and total phenols, showed less efficacy against all the tested bacteria, as pointed out by the higher MBC values.

Keywords: antibacterial activity, rosemary, non-volatile phenolic compounds, disk diffusion method, minimal bactericidal concentration

Introduction

Many infections diseases are known to have been treated with herbal remedies throughout the history of mankind and even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries [1, 2]. *Rosmarinus officinalis* L. (Labiatae), a perennial aromatic herb native to the Mediterranean area and widely distributed in all western Mediterranean countries, is known for its many uses in food and for its pharmacological properties. The volatile fraction and the phenolic constituents are primarily responsible for the biological activity of this plant [3]. To date rosemary is widely used to obtain essential oils and to prepare phenolic leaf extracts that are increasingly employed to provide a natural remedies against some common diseases. According to the ESCOP (European Society Cooperative on Phytoterapy), ethanol and aqueous extracts from rosemary leaves are used as coleretic, colagogue, epatoprotective, and antioxidants, but also as light diuretic, antitumor and antiviral products (4). Recently it has been confirmed the *in vivo* antiulcer activity of an ethanol extract of *Rosmarinus officinalis* L. [5]. The derived essential oil from leaves is mainly used for local applications for its balsamic, antispasmodic and anti-inflammatory activities [6, 4]. The large number of properties attributed to the aqueous and hydroalcoholic extracts from the leaves of *R. officinalis* L. are closely related to their phenolic fraction [7]. Two compounds, rosmarinic acid and carnosic acid, are recognized as the main constituent but several minor flavonoids and some oxidized products from carnosic acid are co-present in these extracts and contribute to define their composition [8].

However, there are still few data about the antimicrobial activity of rosemary phenolic extracts and a more deeper investigation about this property is desirable. Antibacterial activity against foodborne microorganisms was related to the terpenoidic fraction recovered by hexane from a commercial rosemary extract [9]. An high antimicrobial activity against both Gram-positive and Gram-negative bacteria, was related to the carnosic acid content in methanol and acetone extracts from rosemary leaves [7]. A synergistic interaction with BHA to inhibit *Escherichia*

coli and *Staphylococcus aureus* growth was demonstrated by a methanol rosemary extract that not only enhanced the antioxidant efficiency but also the antibacterial effect of BHA, allowing a decrease from 4.4 to 17 fold in the amounts of the synthetic compounds used [10].

Although today few data are available on application in this field, rosemary extract for skin-care cosmetics have been used especially thanks to their UV-protective properties, anti-inflammatory and anti-*S. aureus* activities [11]. An antimicrobial effect combined with the antioxidant property would be very useful to employ this plant in cosmetic preparations.

In this study we investigated on the phenolic composition of three different extracts from rosemary leaves to evaluate and compare their antimicrobial action against two Gram-negative bacteria, *Escherichia coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 15442), and two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 82221). These species are usually required to evaluate the bactericidal activity of common chemical disinfectants [12]. The ability to inhibit the grow of *S. epidermidis* (ATCC 82221) was also tested to explore a possible application of these extracts in cosmetics industry. The aim of our work was to verify the possible correlations between the phenolic composition of three chemically characterized extracts and the antimicrobial activity, evaluated by MBC assay, in inhibiting common saprophytes and commensal microbes. All the phenolic extracts obtained from fresh leaves of rosemary, were characterized in terms of rosmarinic acid, terpenoids and flavonoids by means of HPLC/DAD/MS.

Results and Discussion

Phenolic composition of the rosemary extracts

Aim of this work was to evaluate the antibacterial activity of three different phenolic extracts (E-YL, E-ML and E-F) obtained from fresh rosemary leaves applying an ethanol extraction as previously described [8]. To better understand and

recognize the compounds mainly responsible of the antimicrobial effects, the pure standards, rosmarinic acid and carnosic acid, were also evaluated. These two molecules have been selected because recognized as more abundant constituents of the phenolic fraction of rosemary.

The chromatographic profiles in Figure 1 show the phenolic distribution within E-YL, E-ML and E-F extracts and the histogram in Figure 2 is focused to compare the composition in terms of phenolic classes. The compound identification was carried out by the comparison with the retention time of commercial standards, by their UV-Vis spectra and by interpretation of their mass spectra obtained as reported in our previous study [8].

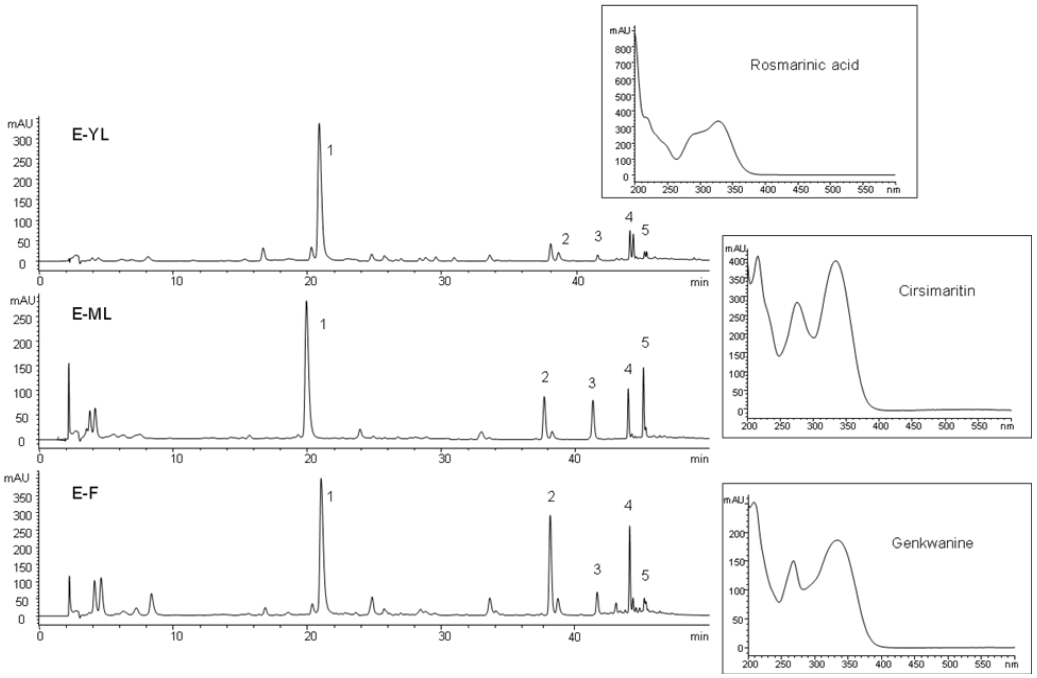

Figure 1. HPLC profiles at 330 nm of the three rosemary extracts (E-YL, E-ML, E-F) and some UV-Vis spectra of the main compounds. *1, rosmarinic acid; 2, cirsimaritin; 3, genkwanine; 4-5, unknown flavonoids.*

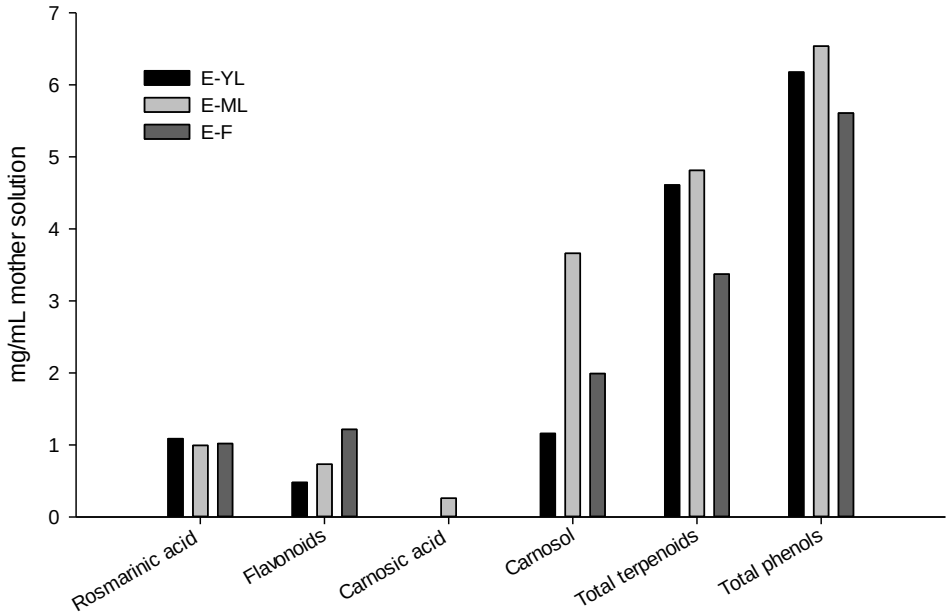

Figure 2. Histogram showing the phenolic composition in the mother solutions of the leaves extract. Each value is a mean of three determinations expressed as mg/mL mother solution, with CV% < 4.9 %.

The E-YL sample from young newly developing leaves of the current-year's growth shows a phenolic profile comparable to that of E-ML extract obtained from mature foliar tissue, characterized by the presence of rosmarinic acid, carnosol and several flavonoids (Figure 1). The E-F extract showed an higher content of flavonoids, because this sample was specifically prepared to better evaluate the role played by this fraction as antimicrobial agents. Large part of these flavonoids are in the aglycone forms; among them the methoxylated flavonoid cirsimaritin has reached concentrations of up to 0.5 mg/mL, being the more abundant together with genkwanin (Figure 3).

The carnosic acid, a typical non-volatile terpenoid of rosemary leaves, was poorly present or absent in our samples mainly due to the extraction from fresh leaves. This finding agrees with literature that reports as in water media the carnosic acid rapidly degrades toward the principal oxidized form carnosol [13], with a consistent decrease after only 24 h at room temperature [14]. Among the tested extracts, the

highest amount of carnosic acid was found in the E-YL extract and the lowest quantity in the E-ML, while an opposite distribution was observed for carnosol. The E-F extract showed the absence of carnosic acid and carnosol and the lowest content in total terpenoids. It can be underlined that cirsimaritin was the major flavonoid in all these extracts, with the maximum amount in the E-F (Figure 3).

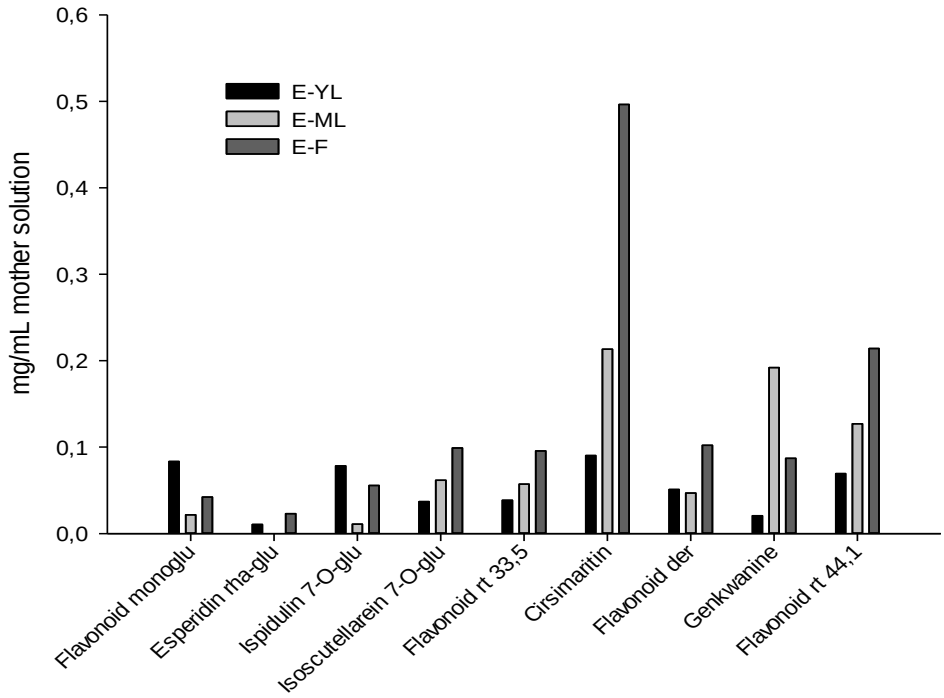

Figure 3. Comparison of the flavonoidic distribution within the three extracts; each value is a mean of three determinations expressed as mg/mL mother solution, with CV% < 4.9%.

Antibacterial activity

The antibacterial activity of rosemary extracts were qualitatively and quantitatively assessed determining the inhibition zones and Minimal Bactericidal Concentration (MBC) as reported in Tables 1 and 2.

Antimicrobial disk susceptibility test was selected as a preliminary procedure for screening the antibacterial efficacy; a limited activity against *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 82221 (Table 1) and no effects at the same concentration

on the *E. coli* ATCC 10536 and *Ps. aeruginosa* ATCC 15442 were observed. The usefulness of this method can be limited by a certain hydrophobicity of the sample that can induce a non homogeneous distribution of the bioactive molecules preventing their uniform diffusion through the agar medium [7]. As previously discussed and according to the chromatographic profiles obtained working with a reverse phase column, the chemical characterization of these extracts confirmed the presence of a group of lipophilic compounds, mainly the terpenoidic fraction and the non glycosilated flavonoids.

Varying degrees of growth inhibition, with differences in the interspecies susceptibility, were observed with these bacterial strains (Table 1).

Samples	**Concentration** (% of mother solution)	***S. aureus* ATCC 6538** (mm)	***S. epidermidis* ATCC 82221** (mm)
E-YL	50	10.3 ± 0.6	13.6 ± 0.9
	75	11.2 ± 0.5	16.0 ± 1.6
	100	12.2 ± 0.9	18.6 ± 0.7
E-ML	50	9.9 ± 1.3	13.2 ± 0.8
	75	10.4 ± 0.8	14.3 ± 0.8
	100	12.6 ± 0.9	17.3 ± 1.2
E-F	50	0.0	13.1 ± 0.6
	75	11.1 ± 0.6	16.3 ± 1.0
	100	12.0 ± 1.2	18.6 ± 1.0
Rosmarinic acid	50	0.0	0.0
	75	0.0	0.0
	100	0.0	0.0
Carnosic acid	50	9.9 ± 1.1	10.0 ± 0.3
	75	10.0 ± 0.6	11.1 ± 0.5
	100	10.0 ± 0.5	12.6 ± 0.7

Table 1. Disk diffusion method results for the rosemary extracts and for rosmarinic and carnosic acids (Values are a mean ± SD. 75% and 50% correspond to dilution 3:1 v/v and 1:1 v/v respectively of the mother solution (100%).

Gram-positive bacteria were more sensitive to the extracts than Gram-negative ones and *S. epidermidis* showed higher inhibition zones than *S. aureus*. The activity of both these bacteria was found to depend on the dosage used when applied at 50%, 75%, or 100% of extract per disk, with inhibition zones ranging from 0.0 to 18.6 mm. The rosmarinic acid did not show inhibition against the selected bacteria in agreement with previous data reported by Moreno *et al*. [7]. On the opposite the

carnosic acid presented a good activity against *S. aureus* and *S. epidermidis*, with inhibition areas close to 10 mm at the lowest concentration (50% of mother solution). *Ps. aeruginosa*, (inhibition of 24.4±0.6 mm with 100 µg of piperacilline) and *E. coli* were not sensitive at all to the three tested extracts. Our phenolic extracts E-YL and E-ML showed the same activity, while for the E-F sample a lower efficacy against the tested bacteria, especially for the *S. aureus*, was observed. Anyway the inhibition zones of well known antibiotics, amoxicillin and piperacillin, were always larger than those of the tested compounds (Table 1).

Table 2 summarizes the results of antibacterial activity tested by the broth microdilution assay expressed as MBC. It was examined the minimum bactericide concentration to evaluate the lowest extract or substance concentration that kills all bacteria, as evidenced by the absence of microbial growth in the corresponding sub-culture after incubation for 48 h. The antimicrobial activity of two pure compounds, rosmarinic acid and carnosic acid, was also tested obtaining the MBC values in Table 2.

	Gram-positive		***Gram-negative***	
	S. aureus **ATCC 6538**	***S. epidermidis*** **ATCC 82221**	***E. coli*** **ATCC 10536**	***Ps. aeruginosa*** **ATCC 15442**
E-YL	130 (8.62)	130 (8.62)	≤ 70 (4.64)	200 (13.3)
E-ML	130 (16.0)	130 (16.0)	≤ 70 (8.6)	200 (24.6)
E-F	270 (28.8)	130 (13.9)	≤ 70 (7.48)	200 (21.4)
Rosmarinic acid	600	600	400	400
Carnosic acid	270	200	≤ 70	270

Table 2. Antibacterial activity (MBC) of the three extracts of *Rosmarinus officinalis* L. and two pure standards (std). The data are expressed as µg /mL of mother solution; the corresponding mg of dried extract are in brackets. The µg/mL of two pure standards are also reported as reference compounds.

On the opposite of the disk diffusion method, *E. coli* was the more susceptible strain against all the extracts (MBC ≤ 70 μg/mL) and also against the two pure standards, carnosic acid (MBC ≤ 70 μg/mL) and rosmarinic acid (MBC = 400 μg/mL). Moreno *et al.* [7] reported the sensitivity of *E. coli* to carnosic acid but the resistance against rosmarinic acid; this latter compound did not show inhibition against this bacterium. In our experiments the standard carnosic acid is more effective especially against *E. coli*, while higher concentrations have been requested for the MBC of the other bacteria (MBC= 200-270 μg/mL). As well as we obtained with disk diffusion method, *Ps. aeruginosa* showed more resistance to these extracts having the higher concentrations (MBC = 200 μg/mL) for E-YL and E-ML than the other bacteria.

As expected, *S. aureus* and *S. epidermidis* have similar sensitivity against the first two extracts in Table 2, having similar phenolic composition and terpenoid content (Figure 2). From our data it emerges that the flavonoids and particularly the high content in cirsimaritin (Figure 3) have not improved the antimicrobial efficacy against these microorganisms. According to the values in Table 2, the E-F, containing the highest concentration of flavonoids but the lower content of terpenoids and total phenols, showed less efficacy against *S. aureus*.

Our results (Table 2) are not completely in agreement with some authors which have pointed out that Gram-positive bacteria are more sensitive than Gram-negative ones, especially in the case of extracts containing carnosic acid as major phenolic compound [15]. In agreement with Moreno *et al*. [7], also in our study no correlations between MBC values and the inhibition zones, obtained by disk diffusion method, have been found. Overall, all the samples tested by broth dilution method have shown an higher activity compared to what obtained by the agar disc diffusion method that seems not to be a suitable approach to study these complex herbal extracts.

Our preliminary data suggest to better investigate on possible synergistic effects between these phenolic components and some common preservatives used in cosmetic and food preparations. Any positive results could promote their use as natural additives for inhibiting microbial growth, especially pathogens.

Experimental

Preparation of the rosemary extracts

Different foliar tissue samples were collected at the same elevational and horizontal position in the plant following the sampling procedure indicated by Squillace [16]. Young newly developing leaves of the current-year's growth, and mature of previous-year old foliar samples were separated in the laboratory. The leaves (1 g) were ground in liquid nitrogen and extracted with ethanol (two steps) by the alternation of magnetic stirring and sonication in ultrasounds, as already described in a previous study [8]. A final liquid/liquid extraction with hexane (1:1, v/v) was applied mainly to remove part of the chlorophylls. To be able to separate the two phases the hexane was used after an addition of a small amount of water (3% of the total volume). The residual ethanol solutions were directly analyzed by HPLC/DAD/ESI/MS. The final samples were E-YL from young newly developing leaves of the current-year's growth and E-ML from mature of previous-year old foliar samples. To obtain the E-F sample, particularly rich in flavonoids, the final ethanol extract after hexane was dried and treated with CH_2Cl_2 to dissolve the more lipophilic components. This latter solution was recovered after centrifugation, dried and re-dissolved in ethanol for the HPLC analyses.

HPLC/DAD/ESI/MS analyses

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (all from Agilent Technologies, Palo Alto, CA, USA).
A 150 mm × 2 mm i.d., 4 μm Fusion, RP18 column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN; the multi-step linear solvent gradient used was: 0–15 min 15–25% B; 15–25 min, 25–35% B; 25–35 min 35–50% B; 35–40 min 50–100% B with a final plateau of 8 min at 100% B; equilibration time 10 min; flow

rate 0.2 mL min^{-1} and oven temperature 26°C; injection volume 5 µL. The analysis conditions were the same described in a previous study [8].

Determination of phenols content

The quantitative evaluation of the main constituents was performed through the use of two external standards, rosmarinic acid at 330 nm and carnosic acid at 284 nm. The first compound was used at 330 nm, to quantify also all the flavonoids, while the second one at 284 nm to determine all the other diterpenoids. The calibration curve of rosmarinic acid (Sigma-Aldrich) was in a linearity range between 0.1 µg and 9.4 µg with a r^2 0,9999; the calibration curve of carnosic acid (Sigma-Aldrich) was in the linearity range of 0.05-3.4 µg with r^2 0.9998.

Microorganisms

All the bacterial strains tested were acquired from the American Type Culture Collection: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 82221), *Escherichia coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 15442).

The stock cultures were preserved in Muller Hinton Agar (MHA-Oxoid Limited) slant at 4°C and sub-cultured every two months. The cultures were prepared by inoculating a loopful of each microorganism in 5 ml of Muller Hinton Broth (MHB-Oxoid Limited) from MHA slant. Broths were incubated at 37°C for 24 hours. The suspension for each microorganism was diluted with physiological solution to obtain about 10^8 ufc mL^{-1} valued by biophotometer (Eppendorf BioPhotometer) (OD 0.200 nm).

Antibacterial Assay

The four bacterial cultures of both Gram-positive and Gram-negative bacterial strains were used for antibacterial activity on *Rosmarinus officinalis* L. using disk diffusion method and broth microdilution assay.

The concentration of the dried extract in the mother solutions, prepared using DMSO, are reported in Table 3.

Antimicrobial disk susceptibility tests. The extracts were tested for antibacterial activity by the disk diffusion method according to the standard procedure of the Clinical and Laboratory Standards Institute [17]. To standardize the inoculum density for a susceptibility test, it was used a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard, so homogeneous cultures containing 10^8 ufc were spread on MHA. Standard 6 mm paper disks (International PBI srl) were placed on the inoculated surface of the agar. The stock solution of the extract was prepared by dissolution in dimethyl sulfoxide (DMSO) of an emulsifier (1mg/1mL). Then, paper discs were individually impregnated with 20 µL of the solution: the tested concentrations were 100%, 75% and 50% evaluated with respect to the mother solutions (each 1 mg/mL of rosmarinic acid). This test was performed as screening and for this reason we used only three concentrations. Standard antibiotic disks were used as positive controls, ampicillin (10 µg/mL) for *E. coli*, *S. aureus*, *S. epidermidis* and piperacillin (100 µg/mL) for *Ps. aeruginosa*. For the negative control DMSO was used. The Petri dishes were kept at 37°C and incubated 24 h. After incubation, all plates were observed for zones of growth inhibition and the diameters in millimeters of these zones were measured. Each assay was performed in triplicate, and the results were expressed as mean ± SD.

Broth microdilution assay (MBC-Minimal Bactericidal Concentration). The MBC (the lowest extract concentration at which no microbial growth was detected) were determined by broth microdilution assay. 20 µL of MHB (Muller Hinton Broth-Oxoid Limited) with 0,5% Tween 80 were added to threefold serial dilutions, and were performed serial dilutions (serial dilution = 600-70 µg/mL) of our extracts and of the two standards (carnosic and rosmarinic acids). In each well were added 20 µL of bacterial suspension (ca.1×10^5 ufc mL^{-1}). After incubation (37°C for 24h) an aliquot (60 µL) of each well was inoculated into plates containing MHA (Muller Hinton agar-Oxoid Limited). Plates were incubated for 24 h at 37°C, and then the MBC values were calculated.

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Ultrasound and microwave techniques provide new insights into phenolics composition of rosemary extracts

Unpublished Results

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Abstract

Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are today a conceivable reality beyond lab-scale procedures. These techniques can be considered a green process as it helps to greatly accelerate the extraction process and reduce energy consumption. Aim of this work was to investigate efficiency and selectivity of UAE and MAE to recover the phenolic fraction from rosemary leaves by means of last-generation devices. Different sequential procedures were applied to investigate on the possibility to improve the total extraction yields, to selectively recover the terpenoidic compounds and to preserve the carnosic acid from the oxidative degradation.

The phenolic content in ethanol under MAE and UAE was more than three times higher than a classic solid-liquid extraction, that requires a duration of extraction ten times higher. Ethanol and acetone seem to be the most suitable solvents for the recovery of phenolic compounds. The water extracts showed the lowest content of total phenols confirming the relatively poor extractive capacity of this solvent. The highest content in rosmarinic acid was obtained in ethanol by UAE (67.7 mg/g dried extract). By comparison of phenolic fractions and final yields it was pointed out that high-intensity US is the most effective and versatile method looking for scaling up. Moreover the findings of our work pointed out that the use of ultrasounds helps to avoid the oxidation processes that produce carnosol and rosmanol, from their precursor carnosic acid.

Keywords: Ultrasound assisted extraction, microwave assisted extraction, solvent, natural products

Introduction

Several traditional methods have been used to extract antioxidants from aromatic and officinal plants, such as conventional solvent extraction (Almela *et al.*, 2006; Doolaege *et al.*, 2007), solid-liquid extraction, aqueous alkaline extraction, extraction with vegetable oils (Señorans *et al.*, 2000), among others.

The design of more efficient extraction processes, that may address the requirements of process intensification and energy saving, has been an important research topic in recent years. Safety, sustainability, environmental and economic factors are all forcing industries to turn to non-conventional technologies and greener protocols (Chemat *et al.*, 2012). Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are today a conceivable reality beyond lab-scale procedures (Cravotto *et al.*, 2011). UAE can be considered a green process as it helps to greatly accelerate the extraction process and reduce energy consumption. The method is clean, and thanks to the low bulk temperature and the rapid execution, preserve the extract from thermal degradation. It leaves no residue in the extract and uses no moving mechanical parts, preventing the occurrence of any pollution. It also offers advantages in terms of productivity, yield and selectivity, improves processing time, enhances quality, reduces chemical and physical hazards (Chemat *et al.*, 2011). Although scarcely reported in the scientific literature (Vinatoru, 2001), since the '90s industrial applications were available with batch reactors from 100 up to 500 L mainly in the preparation of extracts for the phyto-pharmaceutical, cosmetic and liqueurs industry. Extraction enhancement by this technique has been attributed to the propagation of US pressure waves, and resulting cavitation phenomena. High shear forces cause increased mass transfer of extractants (Jian-Bing *et al*., 2006).

In recent years, Albu *et al*. (2004) investigated the effect of different solvents and US on the extraction of carnosic acid from rosemary. Using conventional stirred extraction, ethanol was significantly less effective then ethyl acetate and butanone. The application of US improved the relative performance of ethanol such that it was comparable to butanone and ethyl acetate alone. Thereby high-intensity US may

reduce the dependence on a solvent (Cravotto *et al.*, 2004) and enable use of alternative solvents which may provide more attractive economics, environmental and health and safety benefits. The US devices can be designed in a batch mode as bath, immersion horn or cavitating tube (Figure 1), however for larger-scale work is conceivable in a continuous mode where multiple units can be combined in a sequential manner (loop reactors), which also increases residence time (Figure 2).

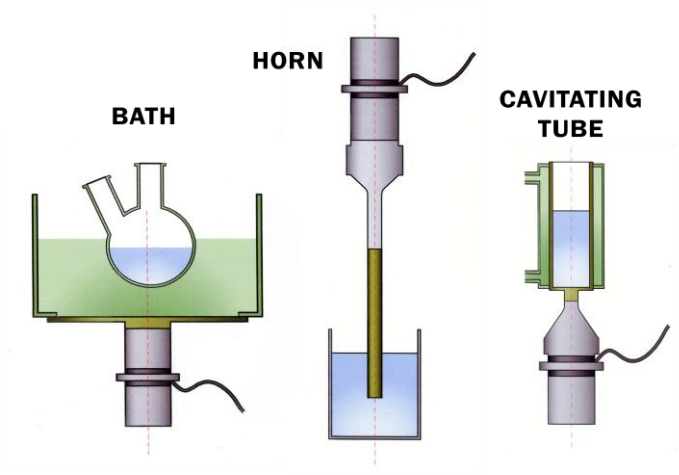

Figure 1. Ultrasonic probe systems (University of Turin and Danacamerini s.a.s. – Turin, Italy)

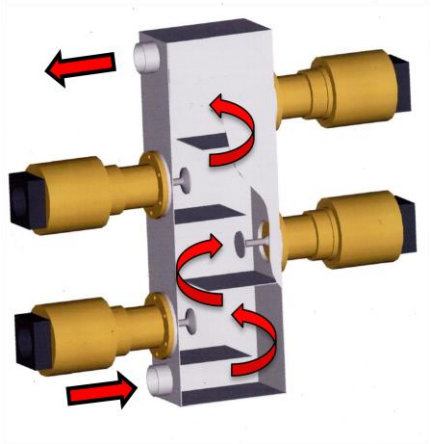

Figure 2. Multi-horn flow reactor (University of Turin and Danacamerini s.a.s. – Turin, Italy)

Flow UAE is a novel achievement in the field which exploits mainly multi-transducer probe systems either fixed on emitting surfaces or equipped with horns. Normally the flow-reactors geometry used in UAE can be easily cleaned online in a very efficient and rapid way and enable easier scaling up (Alexandru *et al.,* 2013).

In recent years also MAE has drawn significant research attention in various fields, in particular medicinal plant research, due to its unique heating mechanism, moderate capital cost and its good performance under atmospheric conditions (Chemat & Cravotto, 2013).

The main advantage of MAE resides in the performance of the heating source. The fast volumetric microwave (MW) heating reduces dramatically both the extraction time and the volume of solvent required. This technique has been continuously improved and compared with classic and non-conventional extraction procedures (Orio, 2012). Besides the fundamental closed (sealed-vessel above atmospheric pressure) and open system for MAE (Dean *et al.*, 2000; Luque-García *et al.*, 2003 respectively), throughout the last decade many modifications have been introduced to enhance its performance. New dedicated MW reactors have been developed to carry on vacuum microwave-assisted extraction (VMAE), nitrogen-protected microwave-assisted extraction (NPMAE), ultrasonic- microwave-assisted extraction (UMAE) and dynamic microwave-assisted extraction (DMAE). High working pressure and temperature accelerate extraction process, improving yields with lower solvent volumes. The pressure inside the extraction vessel is controlled in such a way that it would not exceed the working pressure of the vessel while the temperature can be regulated above the normal boiling point of the extraction solvent. In open systems the upper part of the vessel is connected to a reflux unit to condense any vaporized solvent. Thanks to recent technological advances analytes recovery and reproducibility of MAE are dramatically improved offering an irreplaceable tool for plant extraction. The scheme depicted in Fig. 3 showed a simple device designed for fast and eventually sequential extraction with different solvent under MW irradiation under gas pressure followed by rapid filtration.

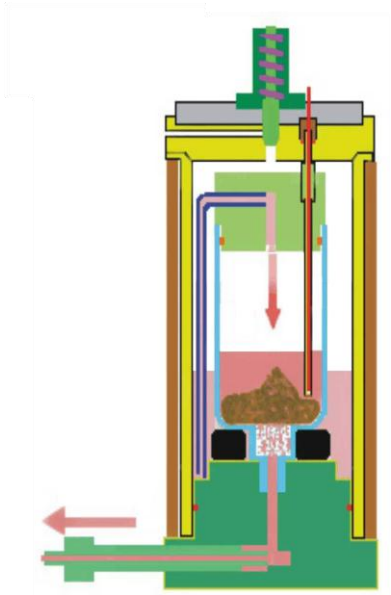

Figure 3. MAE and fast extract solution recovery under inert gas pressure at high temperature (kind permission of MLS GmbH - Leutkirch, Germany)

Aim of this work was to investigate efficiency and selectivity of UAE and MAE to recover the phenolic fraction from rosemary leaves by means of last-generation devices. The extraction time of 10 minutes was applied for all the tests to verify the ability of these innovative techniques to obtain higher yields in a very short time comparing to the traditional methods. Different sequential procedures were applied to investigate on the possibility to improve the total extraction yields, to selectively recover the terpenoidic compounds and to preserve the carnosic acid from the oxidative degradation. By comparison of phenolic fractions and final yields it was pointed out that high-intensity US is the most effective and versatile method looking for scaling up.

Experimental section

Materials and methods

The same batch of leaves of *Rosmarinus officinalis* L., dried at room temperature in dark for some days, were used for the different extractions.

All the extractive procedures applied in this work are summarized in Table 1. Most of the tests have been performed by UAE because, to date, this technique is more easy and less expensive to be applied in a scale-up process with respect to MAE.

For all the extractions, the same extractive ratio, 1 g of dried leaves/10 mL of solvent, and the same extraction time of 10 minutes were applied.

UAE was carried out by means of a probe system equipped with a titanium horn (Danacamerini - Turin) working at 19.5 kHz (power 150 W). MAE was performed in a close multimode reactor (Synthwave, Milestone Bergamo) under N_2 (20 bar) at 100° C.

	Samples	Yields (%)
MW	MW-H_2O	18.04
	MW-EtOH	20
	MW-EtOH70%	18.97
US	US-EtOH	18.73
	1-US-Ace	15.12
	1-US-EtOH	8.3
	1-US-H_2O	22.02
	2-US-Ace	13
	2-US-H_2O	21.4
	3-US-H_2O+βCD	30
	4-US-Hex	6.06
	4-US-Ace	11.43
	4-US-EtOH	6.43
	4-US-H_2O	25.69
	5-US-H_2O	21.43
	5-US-EtOH	17.33
	6-US-Hex	6.59
	6-US-H_2O	21.41
	6-US-EtOH	13.11

Table 1. Extraction procedures carried out on rosemary leaves. The same extractive sequence is indicated by the same number (1-6) and are reported in the applied sequential order: H_2O (water); EtOH (ethanol); EtOH 70% (ethanol/water 7:3 v/v); Ace (acetone); Hex (*n*-hexane); βCD (β-cyclodextrin 1.5% in water)

Different sequential extractions were applied. In the sequence indicated as 1, acetone has been used in the first extraction step followed by ethanol (second step) and water for the last extraction cycle. The same was done for the other extraction sequences, as described in Table 1.

In the sample 3, a single extraction step was performed, namely UAE in a 1.5% β-cyclodextrin solution in water.

For the preparation of the reference phenolic extract "standard extract", leaves (1 g) were dipped in liquid nitrogen and immediately finely grounded in a lab mill. The powder was extracted twice with ethanol alternating magnetic stirring and sonication, as already described in a previous study (Mulinacci *et al*., 2011). In addition, a commercial oleoresin powder from rosemary leaves (provided by Giotti S.p.A.) has been considered and analyzed.

All the extracts and the oleoresin were dissolved in a defined volumes of solvent and the solutions were directly analyzed by HPLC.

HPLC/DAD analyses

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (all from Agilent Technologies, Palo Alto, CA, USA).

A 150 mm × 2 mm i.d., 4 μm Fusion, RP18 column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN; the multi-step linear solvent gradient used was: 0–15 min 15–25% B; 15–25 min, 25–35% B; 25–35 min 35–50% B; 35–40 min 50–100% B with a final plateau of 8 min at 100% B; equilibration time 10 min; flow rate 0.2 mL min^{-1} and oven temperature 26°C; injection volume 5 μL. The analysis conditions were the same described in a previous study (Mulinacci *et al*., 2011).

Quantitative determination

The quantitative evaluation of the main constituents was performed through the use of two external standards, rosmarinic acid at 330 nm and carnosic acid at 284 nm.

The first compound was used at 330 nm, to quantify also all the flavonoids, while the second one at 284 nm to determine all the other diterpenoids. The calibration curve of rosmarinic acid (Sigma-Aldrich) was in a linearity range between 0,1 μg and 9,4 μg with a r^2 0,9999; the calibration curve of carnosic acid (Sigma-Aldrich) was in the linearity range of 0,05-3,4 μg with r^2 0,9998.

Results and Discussion

Extraction efficiency

Table 2 reports the phenolic compounds content of 20 different extracts obtained applying different extraction techniques.

Method	Rosmarinic acid	Flavonoids	Terpenoids	Total phenols
MW-H_2O	6.20	2.42	-	8.62
MW-EtOH	5.11	3.05	28.47	36.63
MW-EtOH70%	6.24	3.38	25.28	34.90
US-EtOH	3.71	2.96	28.34	35.02
1-US-Ace	1.30	2.46	31.01	34.77
1-US-EtOH	3.86	1.47	7.92	13.25
1-US-H_2O	4.78	10.28	-	15.06
2-US-Ace	1.13	2.42	28.90	32.45
2-US-H_2O	-	2.77	-	2.77
3-US-H_2O+βCD	-	5.39	-	5.39
4-US-Hex	-	0.46	11.86	12.32
4-US-Ace	2.00	2.07	18.33	22.40
4-US-EtOH	4.35	1.61	1.98	7.94
4-US-H_2O	-	11.93	-	11.93
5-US-H_2O	-	2.72	-	2.72
5-US-EtOH	-	7.47	32.11	39.58
6-US-Hex	-	0.55	12.62	13.17
6-US-H_2O	-	3.96	-	3.96
6-US-EtOH	-	3.01	19.91	22.92
Standard extract	0.56	1.6	8.12	10.28

Table 2. Amount of the main phenolic constituents in the extracts obtained using different extraction procedures. Data are expressed as mg/g DW. *Standard extract: a reference phenolic extract obtained by conventional extraction procedure (Mulinacci et al., 2011); -, not detected*

Figure 1 reports the extraction efficiency of different techniques and conditions expressed as content of total phenol (mg/g dried leaves); US1, US2, US4, US5 and US6 columns are the sum of the various sequential extraction steps on the same foliar sample and show the efficiency in terms of total phenolic recovery.

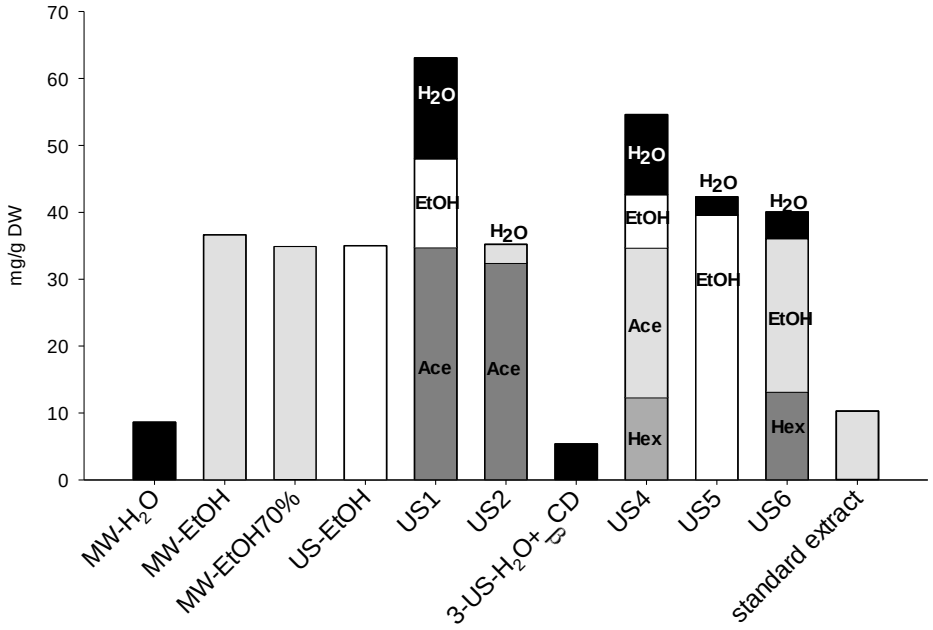

Figure 1. Total phenolic content (mg/g dried weight) of all the extracts under MAE and UAE

As showed in Figure 1 and Table 1, the phenolic content under MAE and UAE was more than three times higher than a classic maceration "Standard extract", a solid-liquid ethanol extraction that requires at least two hours of extraction time. This traditional method has proved a reduced efficacy compared with the ethanol extracts obtained applying these innovative techniques. Nevertheless, the solvent clearly plays a major role on extraction performance. In rosemary leaves extraction ethanol and acetone seems to be the most suitable solvents for the recovery of phenolic compounds. In sequential extraction procedures in MAE and UAE, water confirms its relatively poor extractive capacity when used after organic solvents (US1, US2, US4 and US6). Anyway even when water was used in the first run, low yields were

observed (US5). Regarding the use of ethanol, the results were very similar applying US and MW (US-EtOH, MW-EtOH); the two extraction methods showed comparable phenolic content with 35.02 and 36.63 mg/g DW, respectively, as shown in Table 2.

Moreover, when the extraction by ethanol was performed as second (US1, US6) or third (US4) step, it was less effective and the recovery of phenols the lowest. On the opposite, this trend is not observed in the sample 5-US-EtOH where ethanol extraction followed a first extraction step with water. The sequences US1 and US4 presented the highest concentration of total phenols showing that after acetone, the ethanol and also water has been proven to be efficient for recovering the residual phenolic compounds from leaves. Overall the application of these two extractive sequences can increase the total efficiency of the process, while the other two sequences US5 and US6 are less suitable for recovering all the phenolic components.

Focusing on the terpenoidic fraction, Figure 2 compares the carnosic acid and total terpenoid contents, expressed as mg/g dried leaves, reported for the extracts containing these compounds; the histogram shows the efficiency of thirteen extraction methods and highlights the samples richer in carnosic acid.

Overall the most efficient extraction, considering both the total terpenoid and carnosic acid contents, was obtained for the sample 1-US-Ace (3.1% on dried leaves) with comparable amounts, even if a little bit lower, for the samples US-EtOH, MW-EtOH and 2-US-Ace ranging from 2.83% to 2.85%.

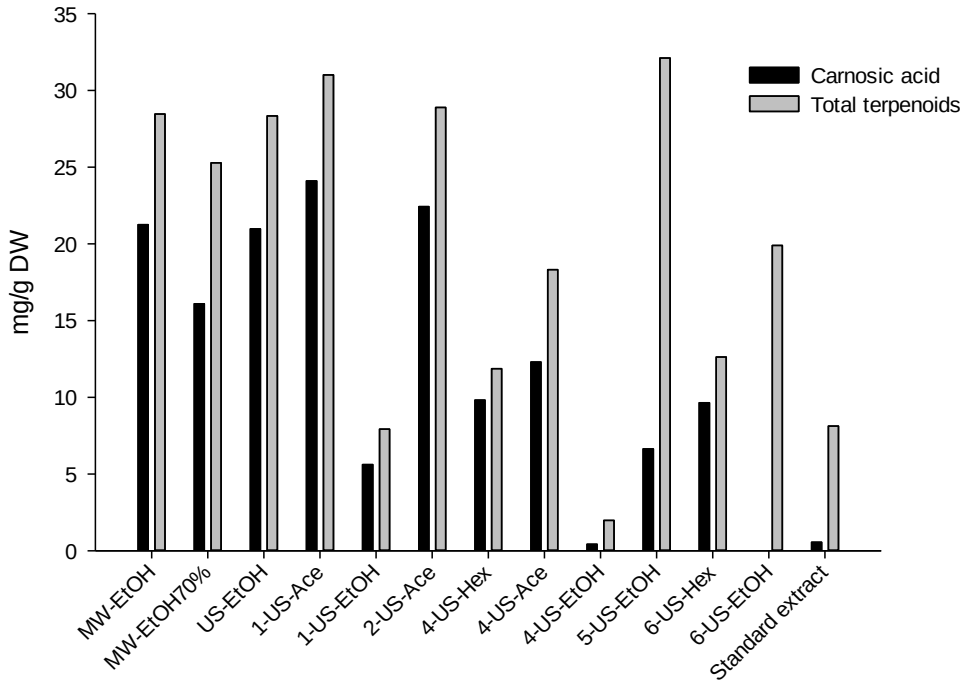

Figure 2. Amount of terpenoids (mg/g dried leaves) of the richer extracts obtained under MAE and UAE

In the light of these findings it can be assessed that the acetone (1-US-Ace and 2-US-Ace) has proved the best efficacy, and its use has allowed to obtain the highest yields in terms of terpenoids from dried leaves; this solvent was also able to recover high amount of total phenols (3.48% and 3.25%, respectively), as also reported in Table 2.

Regarding the use of ethanol, also considering the total content of terpenoids, the results were similar for the two extraction techniques (US-EtOH, MW-EtOH); MAE and UAE methods showed comparable amounts in the content of terpenoids (28.34 and 28.47 mg/g DW, respectively).

Very low concentrations of terpenoids have been found in the extracts 1-US-EtOH and 4-US-EtOH, pointing out that the use of ethanol after a pretreatment with acetone, or with hexane and acetone, has not been useful to recover these

molecules; in fact the terpenoidic compounds were exhaustively recovered with the previous extraction (1-US-Ace and 4-US-Ace, Figure 1).

Interesting findings were obtained with *n*-hexane under UAE presenting a good reproducibility (4-US-Hex and 6-US-Hex can be considered as replicates with 1.23% and 1.32% total phenols yields respectively). Although the total phenols concentration was about three times lower than acetone and ethanol extracts, *n*-hexane showed a higher selectivity. As shown in Figure 3, these extracts contained only terpenoids and traces of lipophylic flavonoids. This consistently differ from traditional *n*-hexane liquid/solid extraction where the non-volatile terpenoids cannot be recovered.

Recently other authors proposed the use of SFE, using CO_2 and ethanol as co-solvent, to obtain a fraction with a similar composition of our *n*-hexane US extracts (carnosic acid, carnosol, genkwanine and cirsimaritin) (Herrero *et al.*, 2010). Moreover, in a scale up process, the application of the US extraction is an easier and more convenient process if compared with the instrumentation required for SFE technique.

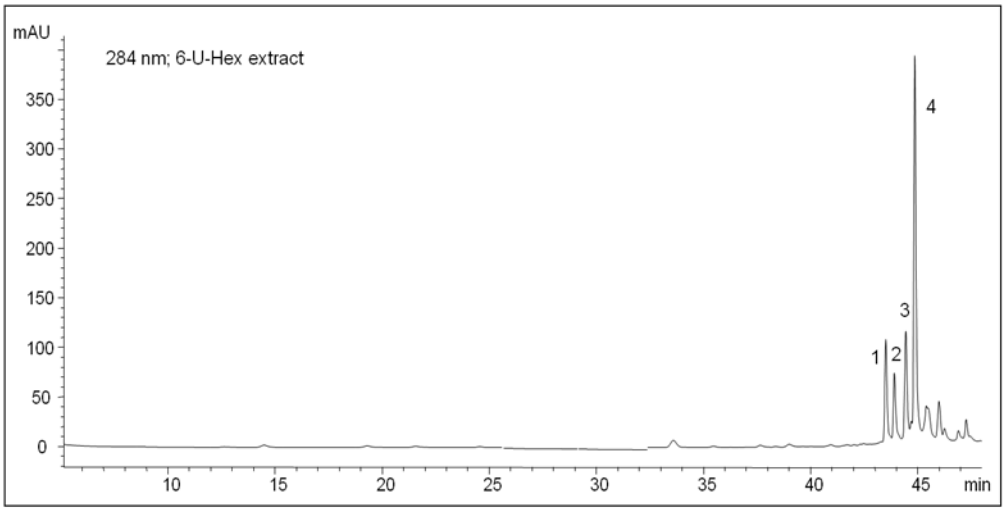

Figure 3. HPLC profile at 284 nm of the 6-US-Hex sample from rosemary leaves. *1, flavonoid; 2, carnosol; 3, flavonoid; 4, carnosic acid*

As reported in Figure 2, the carnosic acid content in 4-US-EtOH and in "Standard extract" samples was very low and even absent in 6-US-EtOH extract.

Moreover the sample 5-US-EtOH, although characterized by the highest amount of total terpenoids, showed a low concentration of carnosic acid, not over 20.6% of the total terpenoid content. This is not unexpected because the ethanolic extraction was applied after a previous treatment with water (5-US-H_2O sample) that can promote the oxidation of carnosic acid towards carnosol, as already described in previous studies (Masuda *et al*., 2002; Zhang *et al.,* 2012). It has been demonstrated that low amounts of water during the extraction may be able to strongly reduce the content of carnosic acid promoting its conversion into oxidation products.

Moreover, the HPLC profile of the sample 5-US-H_2O highlighted that the use of water in US extraction could not recover neither the more polar phenolic fraction nor the rosmarinic acid and the final yields in term of total phenolic content were only 0.27% with respect to the dried leaves.

Quality of the extracts

The content of specific marker compounds or the total phenolic amount expressed as w/w on the dried extracts can be considered useful parameters to evaluate the extract quality.

In Table 3 are compared the phenolic compositions of the eight samples that showed a total phenolic content over 120 mg/g dried extract and of a commercial dried oleoresin from rosemary leaves. The highest concentration of total phenols and total terpenoids have been obtained by acetone extraction (1-US-Ace and 2-US-Ace) with values close to those obtained considering the commercial oleoresin. Nevertheless the acetone extracts obtained applying the US technique were considerably richer in carnosic acid (more than 172 mg/g DE) if compared with the oleoresin (31.6 mg/g DE). These findings suggest that US extraction can be applied to avoid the oxidation processes that produce carnosol and rosmanol from their precursor carnosic acid.

	1-US Ace	1-US EtOH	2-US Ace	4-US Ace	4-US EtOH	MW EtOH	MW EtOH 70%	US EtOH	Oleoresin
Flavonoid	0.4	2.6	0.4	0	1.8	0	0	0	0,1
Flavonoid	0.2	0.4	0.2	0.6	4.4	1.2	1.6	1.1	0.2
Rosmarinic acid	8.6	46.5	8.7	17.5	67.7	25.6	32.9	19.8	0.2
Flavonoid	0.3	2.1	0.6	0.7	3.5	1.7	1.2	0.9	0.1
Flavonoid	0.6	2.9	0.5	0.1	1.5	0.7	3.7	0.8	0.1
Flavonoid	1.0	3.3	1.1	1.4	4.1	2.1	1.9	1.6	0.2
Flavonoid	0	0	0	0.2	4.9	0.8	0.9	0.4	0.4
Flavonoid	0	0	0	1.2	1.3	0	0	0.8	0.6
Cirsimaritin	4.2	2.1	4.9	5.1	1.3	3.0	2.7	3.3	1.8
Flavonoid	0.8	0.4	1.3	1.1	0.5	0.5	0.6	0.6	0.5
Genkwanine	2.5	1.1	2.7	3.1	0.7	1.4	1.5	1.7	1.2
Flavonoid	3.8	1.7	4.2	2.9	0.7	2.5	2.5	2.8	0.7
Flavonoid	2.4	1.1	2.7	1.7	0.4	1.5	1.4	1.7	0.7
Total flavonoids	16.3	17.7	18.6	18.1	25.1	15.3	17.8	15.8	6.8
Rosmanol	10.1	4.8	11.2	11.3	0	6.6	0	7.5	70.5
Carnosol	35.6	22.9	38.5	41.6	24.2	29.4	48.4	31.8	122.3
Carnosic acid	159.4	67.7	172.4	107.5	6.5	106.3	84.9	112.0	31.6
Total terpenoids	205.1	95.5	222.1	160.4	30.8	142.3	133.3	151.3	224.3
Total phenols	230.0	159.6	249.4	195.9	123.5	183.2	184.0	187.0	231.2

Table 3. Phenolic compositions (mg/g dried extract) of the eight samples having an high total phenolic content (over 120 mg/g dried extract) and containing all the main phenols of rosemary leaves. A commercial dried oleoresin from rosemary leaves was also used to compare the obtained data

The highest content in rosmarinic acid was obtained in ethanol under UAE (4-US-EtOH and 1-US-EtOH, 67.7 mg/g and 46.5 mg/g, respectively). Lower amounts have been observed with the same solvent under MAE (MW-EtOH 25.6 mg/g and MW-EtOH 70% 32.9 mg/g).

Our results pointed out a greater ability to recover the rosmarinic acid when the ethanol is used as extraction solvent; this trend is particularly evident when ethanol follows the pre-extraction with acetone. Under MAE conditions the use of a mixture ethanol/water 7:3 v/v, resulted more efficient for rosmarinic acid (yield 28.5%) than the use of pure ethanol (Table 3).

Figure 4 shows the phenolic composition of the four extracts containing mainly terpenoids, together with a group of minor flavonoids. The high amount of terpenoids found in the *n*-hexane extracts (4-US-Hex and 6-US-Hex) was close to 200 mg/g DE. Moreover, as already observed, this solvent afforded a high content of carnosic acid, while ethanol (5-US-EtOH) showed carnosol as main compound (79.5% of total terpenoids). The *n*-hexane samples contained only two lipophilic flavonoids with a total amount ranging from 3.9% to 4.2% of the total terpenoids, as also reported in Figure 3.

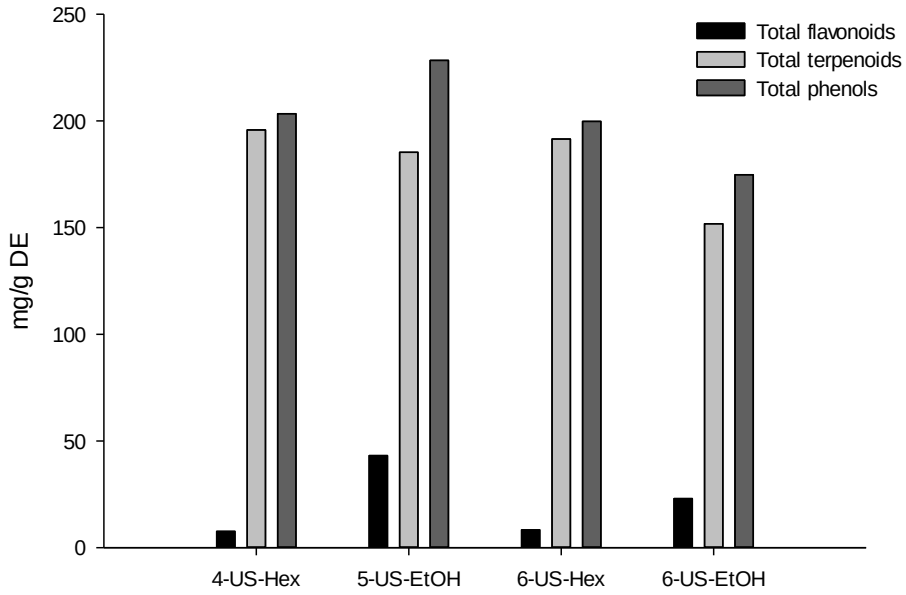

Figure 4. UAE extracts characterized by a good selectivity for the recovery of the terpenoidic fraction

The water extracts obtained by MW and US showed the lowest content of total phenols (<50 mg/g DE) (Table 4). The addition of β-CD was evaluated to verify the possibility to selectively concentrate specific flavonoids from the phenolic fraction. Nevertheless, by the HPLC evaluation, no selective extraction was pointed out and a low recovery of the phenolic molecules, almost exclusively constituted by minor flavonoids, was obtained. Overall, the use of this polar solvent is not recommended for future MW and US extractions on rosemary leaves.

Samples	Rosmarinic acid	Total flavonoids	Total phenols
MW-H_2O	34.36	13.42	47.78
2-US-H_2O	0	12.97	12.97
3-US-H_2O+βCD	0	17.05	17.95
4-US-H_2O	0	46.42	46.42
5-US-H_2O	0	12.71	12.71
6-US-H_2O	0	18.52	18.52

Table 4. Phenolic composition of extract in H_2O and H_2O+βCD extracts under UAE and MAE (mg/g DE)

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The neuropathy-protective action of rosemary in a rat model of neuropathic pain

In cooperation with:
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The effect of different rosemary extracts in the treatment of neuropathic pain was evaluated in the Chronic Constriction Injury (CCI) model of neuropathy (Bennett and Xie, 1988).
Chronic Constriction Injury (CCI) was induced by ligation of the right sciatic nerve (ipsilateral). Fourteen days after injury, the anti-hyperalgesic effect of repeated administration of rosemary extracts has been evaluated by Paw Pressure test, Von Frey test and Incapacitance test.
The tested extracts were:

- an ethanol extract (EE), obtained as described in a previous study (Mulinacci *et al*., 2011)
- an acetone extract (AE), characterized by high concentration of terpenoids (carnosic acid and its derivatives) and by the absence of rosmarinic acid.

The chromatographic profiles of the extracts are shown in Figure 1 and 2.

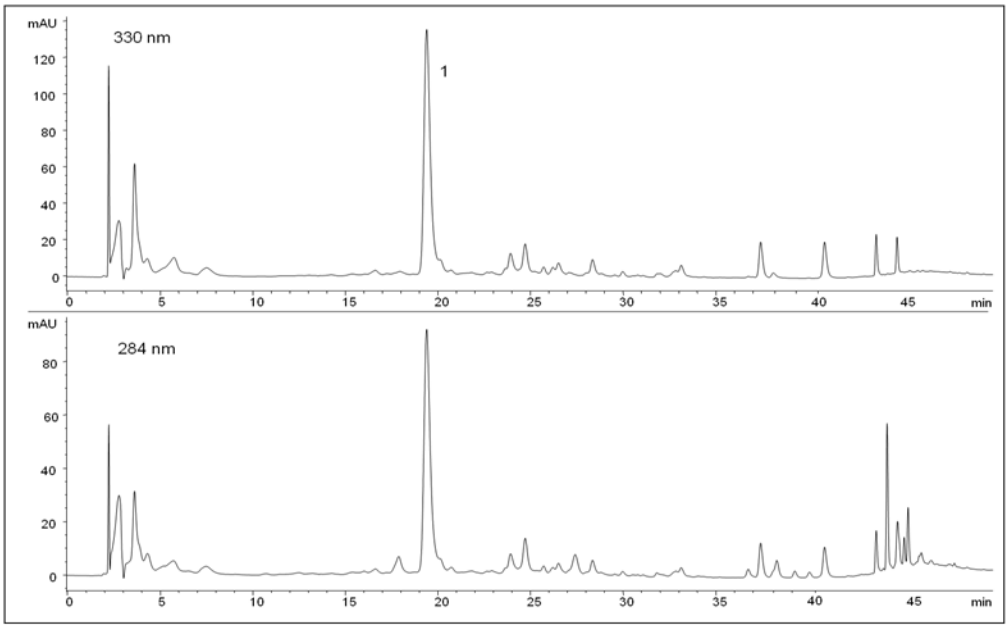

Figure 1. HPLC profile of the ethanol extract (EE) at 330 e 284 nm. Rosmarinic acid (1) is the main compound.

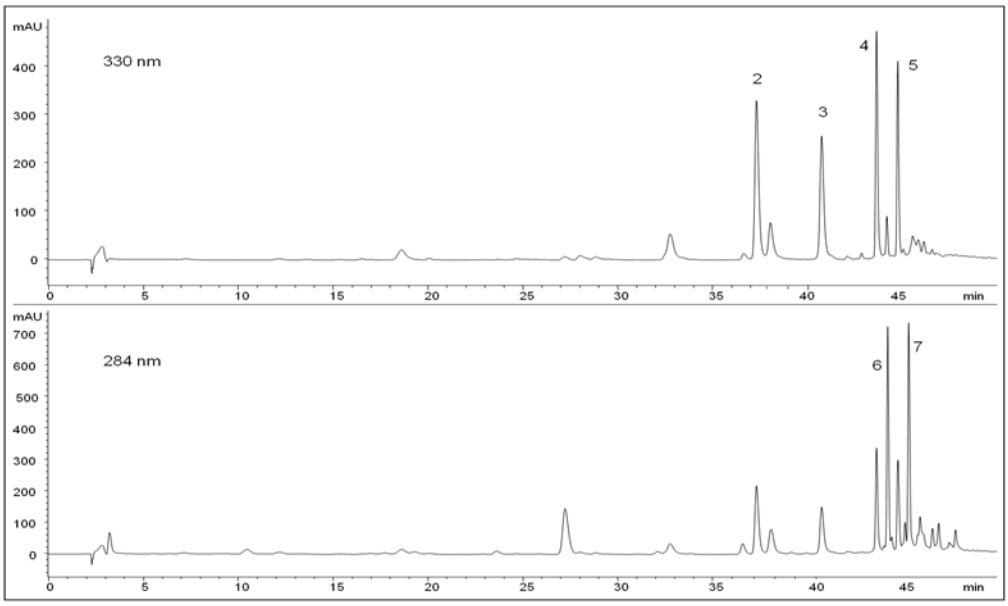

Figure 2. HPLC profile of the acetone extract (AE) at 330 e 284 nm. 2, cirsimaritin; 3, genkwanine; 4-5, unknown flavonoids; 6, carnosol; 7, carnosic acid.

Drug treatments

Rosemary extracts were suspended in 1% carboxymethylcellulose sodium salt (CMC) and administered by the *per os* (p.o.) route. 100 mg kg^{-1} and 300 mg kg^{-1} extracts were daily administered starting from the day 1, immediately after ligation of the sciatic nerve, to the 13th day. Behavioural and biochemical tests were performed 24 hours after the end of treatments on day 14. Control rats received p.o. CMC every day.

Paw pressure test

The nociceptive threshold in rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton *et al.* (1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration (25%) were rejected. An arbitrary cut-off value of 250 g was adopted. Fourteen days after the operation rats were tested twice in 30 min and mean was shown. The data were collected by an observer who was blinded to the protocol.

Von Frey Test

The animals were placed in 20- × 20-cm plexiglas boxes equipped with a metallic meshy floor, 20 cm above the bench. A habituation of 15 minutes was allowed before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying force ranging from 0 to 50 grams with a 0.2 gram accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the meshy floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. Paw sensitivity threshold was defined as the minimum pressure required to elicit a robust

and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each anterior paw with an interval of 5 seconds. The measure was repeated 5 times and the final value was obtained by averaging the 5 measures.

Incapacitance test

Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury [26]. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value considered for each animal was the mean of 5 consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating a postural equilibrium, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. Data are expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one (Δ Weight).

Preliminary Results

14 days after injury (CCI) the mechanical withdrawal threshold to a noxious stimulus was measured by Paw pressure test (Table 3). The weight tolerated on the ipsilateral paw of vehicle-treated animals was significantly reduced (41.2 ± 1.5 g) compared to the contralateral (70.0 ± 2.1 g).

100 mg Kg^{-1} EE and AE administered daily p.o. for 13 days (starting from the day of injury) increased the withdrawal threshold of the ipsilateral paw up to 54.1 ± 1.7 and 58.3 ± 1.2 g, respectively. The higher dose of 300 mg Kg^{-1} did not induce significant increase in pain threshold in comparison with the lower. The acetone extract AE showed higher efficacy than EE also at 300 mg Kg^{-1}.

	Paw pressure		
TREATMENT		Paw	Weight (g)
	Dose mg Kg^{-1} p.o.		*14th day*
Vehicle		ipsilateral	76.2 ± 1.6
		contralateral	73.5 ± 2.9
CCI + Vehicle		ipsilateral	41.2 ± 1.5*
		contralateral	70.0 ± 2.1
CCI + EE	100	ipsilateral	54.1 ± 1.7^
		contralateral	68.5 ± 2.1
CCI + EE	300	ipsilateral	51.2 ± 3.1^
		contralateral	67.5 ± 3.2
CCI + AE	100	ipsilateral	58.3 ± 1.2^
		contralateral	68.0 ± 3.7
CCI + AE	300	ipsilateral	60.0 ± 3.5^
		contralateral	67.5 ± 3.2

Table 3. Effect of rosemary extracts on Chronic Constriction Injury induced hyperalgesia in the rat; PAW-PRESSURE TEST. Paw pressure test was performed on day 14. **P<0.01 with respect to the contralateral paw; ^P<0.01 with respect to the CCI + vehicle treated rats. Each value represents the mean of 10 rats.*

Table 4 shows the response to a non-noxious mechanical stimulus evaluated by the Von Frey test. On day 14 pain threshold of the ipsilateral paw (CCI + vehicle group) was decreased to 9.2 ± 0.9 g as compared to the contralateral (25.7 ± 1.0 g). Animals treated with 100 mg Kg^{-1} EE and AE showed an ipsilateral threshold of 8.4 ± 1.1 and 12.9 ± 0.6 g, respectively; the groups treated with higher dosage tolerated a stimulus by about 15 and 16 g, respectively.

In both Paw pressure and Von Frey tests the pain sensitivity of the contralateral paw of CCI + vehicle or CCI + EE and AE groups was not different with respect to the control (vehicle + vehicle).

	Von frey		
TREATMENT		Paw	Weight (g)
	Dose $mgKg^{-1}$ *p.o.*		14^{th} *day*
Vehicle		ipsilateral	24.6 ± 2.1
		contralateral	29.1 ± 4.3
CCI + Vehicle		ipsilateral	9.2 ± 0.9*
		contralateral	25.7 ± 1.0
CCI + Rostot	100	ipsilateral	8.4 ± 1.1
		contralateral	29.4 ± 3.2
CCI + Rostot	300	ipsilateral	15.2 ± 1.3^
		contralateral	27.4 ± 1.4
CCI + Roscarn	100	ipsilateral	12.9 ± 0.6
		contralateral	29.6 ± 1.5
CCI + Roscarn	300	ipsilateral	16.1 ± 2.0^
		contralateral	28.1 ± 2.3

Table 4. Effect of rosemary extracts on Chronic Constriction Injury induced allodynia in the rat; VON FREY TEST. Von frey test was performed on day 14. **P<0.01 with respect to the contralateral paw, ^P<0.05 with respect to the CCI + vehicle treated animals. Each value represents the mean of 10 rats.*

Unilateral pain was also able to induce hind limb weight bearing alterations (Incapacitance test): the difference between the weight burdened on the contralateral and the ipsilateral limb was significantly increased in CCI + vehicle (79.5 ± 4.6 g) with respect to vehicle + vehicle (-3.9 ± 1.1). The protective effect of rosemary was shown in Table 5.

	Incapacitance test	
TREATMENT		Difference score (g) *contralateral minus ipsilateral paw*
	Dose $mgKg^{-1}$ *p.o.*	14^{th} *day*
Vehicle		-3.9 ± 1.1
CCI + Vehicle		79.5 ± 4.6*
CCI + Rostot	100	81.1 ± 5.2
CCI + Rostot	300	32.9 ± 3.7^
CCI + Roscarn	100	87.5 ± 3.1
CCI + Roscarn	300	23.4 ± 2.8^

Figure 5. Effect of rosemary extracts on Chronic Constriction Injury-induced hind limb weight bearing alterations in the rat; INCAPACITANCE TEST. Incapacitance test was performed on day 14. **P<0.01 with respect to the control animals; ^P<0.01 with respect to the CCI + Vehicle treated animals. Each value represents the mean of 10 rats.*

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LIST OF PUBLICATIONS

PROCEEDING

R. Donato, F. Santomauro, C. Sacco, R. Capei, M. Innocenti, M. Bellumori, N. Mulinacci. ANTIFUNGAL ACTIVITY AGAINST *CANDIDA* SPP. OF PHENOLIC EXTRACTS FROM LEAVES OF *ROSMARINUS OFFICINALIS* L.; poster presented at the ChimAlSi_2012, IX Congresso Italiano di Chimica degli Alimenti, Ischia (Italy), June 3-7th 2012.

C. Sacco, F. Santomauro, R. Donato, R. Capei, M. Innocenti, M. Bellumori, N. Mulinacci. ANTIMICROBIAL ACTIVITYOF PHENOLIC EXTRACTS FROM LEAVES OF *ROSMARINUS OFFICINALIS* L.; poster presented at the ChimAlSi_2012, IX Congresso Italiano di Chimica degli Alimenti, Ischia (Italy), June 3-7th 2012.

Maria Bellumori*, Marzia Innocenti, Francesca Ieri, Lorenzo Cerretani, Mirco Mezzetti, Nadia Mulinacci. THE EFFECTS OF BOILED STEAM COOKING ON RED AND PURPLE FLESH POTATOES: EVALUATION OF ANTHOCYANIN AND PHENOLIC ACID CONTENT; poster (*presenter) presented at the XXVIth International Conference on Polyphenols (ICP), Florence (Italy), July 22-26th 2012.

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