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Molecules of Interest

Carnosic acid

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ABSTRACT

Carnosic acid (salvin), which possesses antioxidative and antimicrobial properties, is increasingly exploited within the food, nutritional health and cosmetics industries. Since its first extraction from a *Salvia* species (~70 years ago) and its identification (~50 years ago), numerous articles and patents (~400) have been published on specific food and medicinal applications of *Rosmarinus* and *Salvia* plant extracts abundant in carnosic acid. In contrast, relevant biochemical, physiological or molecular studies *in planta* have remained rare. In this overview, recent advances in understanding of carnosic acid distribution, biosynthesis, accumulation and role *in planta*, and its applications are summarised. We also discuss the deficiencies in our understanding of the relevant biochemical processes, and suggest the molecular targets of carnosic acid. Finally, future perspectives and studies related to its potential roles are highlighted.

1. Introduction

Well before its chemical structure (Fig. 1; **1**) was elucidated, carnosic acid (salvin) (**1**) and similar compounds of the ferruginol type were extracted from sage *Salvia carnosa* Dougl. as a 'bitter principle' (White and Jenkins, 1942). At that time, probably due to oxidation processes, only a derivative of carnosic acid (**1**) could be isolated and named carnosol (**2**) (pikrosalvin).

Carnosic acid (**1**) was first discovered by Linde in *Salvia officinalis* L. (Linde, 1964). Later, Wenkert et al. (1965) found carnosic acid (**1**) at much higher levels (~3% on weight basis of air-dried leaves) in *Rosmarinus officinalis* L. leaves (Wenkert et al., 1965). Since then, rosemary cultivars highly abundant in carnosic acid (**1**) (4–10% on weight basis of air-dried leaves) were developed, including VAU3, Daregal, Farinole, 4 English, Severn Seas, Miss Jessops Upright, 1 English, Lighthorne culinary (Wellwood and Cole, 2004). The molecule has also been found in other *Salvia* species and other genera of the Lamiaceae (Luis, 1991). Apart from the influence of the genetic background, contents in carnosic acid (**1**) may also be modulated by growth conditions (Tounekti and Munné-Bosch, 2012). Recently, Tounekti and Munné-Bosch (2012) have reviewed certain aspects of phenolic diterpene biology, with a particular focus on the physiological, rather than trans-genetic, approaches, to enhancing and improving the phenolic diterpene levels and composition in *Salvia* and *Rosmarinus* plants and plant extracts. For instance, authors reported that the English climate favours the production of carnosic acid more than the warmer, more arid environmental conditions found in Mediterranean countries where rosemary and sage are typically found. Furthermore, rosemary plants subjected to enhanced levels of UV-B radiation display higher yields of carnosic acid than non-treated plants. Moreover, water, salinity, intense light, and heat stress seem to negatively affect carnosic acid concentrations. Although stress conditions alone seem to decrease levels in carnosic acid (**1**), when applied together with supplements, they result in high yields in phenolic diterpenes. This was confirmed when low amounts of fertilizer or kinetin were supplemented to plants upon saline stress (Tounekti and Munné-Bosch, 2012). Carnosic acid (**1**) is a phenolic diterpene with a formula $C_{20}H_{28}O_4$. It belongs to the largest class of over 50,000 plant secondary metabolites termed terpenoids, also known as isoprenoids or terpenes (Hill and Connoly, 2013). Because carnosic acid (**1**) contains a phenolic group, it is often classified among polyphenols. Yet, its cellular distribution, biosynthetic pathway, solubility properties and roles substantially differ from the majority of polyphenolic classes and rather resemble terpenoids such as tocopherols and carotenoids.

Despite the great interest that this molecule, as part of plant extracts, has received for industrial applications, surprisingly few studies have been conducted into its biology. This overview aims

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to bring together what is known and to highlight deficiencies in our basic knowledge of carnosic acid.

2. Taxonomic, morphological and (sub)cellular distribution of carnosic acid

Mediterranean plants are exposed to a combination of environmental stress conditions, including low water availability, high light, temperature fluctuations, and nutrient deprivation. Such stresses may lead to an imbalance between antioxidant defences and the amount of ROS, resulting in oxidative stress. Besides a number of compounds that have been recognised to protect the chloroplasts from oxidative damage, including carotenoids, α-tocopherol, ascorbate, and glutathione, some plants have evolved carnosic acid, which displays high antioxidant properties *in vitro* and may play a part in the arsenal of antioxidative compounds that contribute to the protection of the chloroplast from oxidative damage. To date, carnosic acid (**1**) has been identified in only a few species, all exclusive of the Lamiaceae (Luis, 1991; Brieskorn and Dumling, 1969; Luis and Johnson, 2005; Bruno et al., 1991; Hossain et al., 2010; Achour et al., 2012; Djarmati et al., 1991) (Fig. 2). To the best of our knowledge, only seven out of 70 genera of the Mentheae tribe contain carnosic acid: *Salvia* (Brieskorn and Dumling, 1969), *Rosmarinus* (Luis and Johnson, 2005), *Lepechinia* (Bruno et al., 1991), *Oreganum* (Hossain et al., 2010) and *Thymus* (Achour et al., 2012). It may be present in *Hyssopus* where one of its possible derivatives, rosmanol-9-ethyl ether (**7**), was identified (Djarmati et al., 1991). Carnosic acid (**1**) also occurs as a minor compound in one genus of the Ocimeae tribe, *Ocimum* (Jayasinghe et al., 2003). Species belonging to the above genera all differ in carnosic acid (**1**) content. Presently, *R. officinalis* is considered to be the most abundant source of carnosic acid, followed by a number of *Salvia* species. Considerable intra-genus and intra-species variations remain regarding carnosic acid (**1**) content. In *Salvia*, 50 out of 62 screened species contained carnosic acid (**1**) with concentrations ranging from 0.1 to 21.8 mg g^{-1} DW

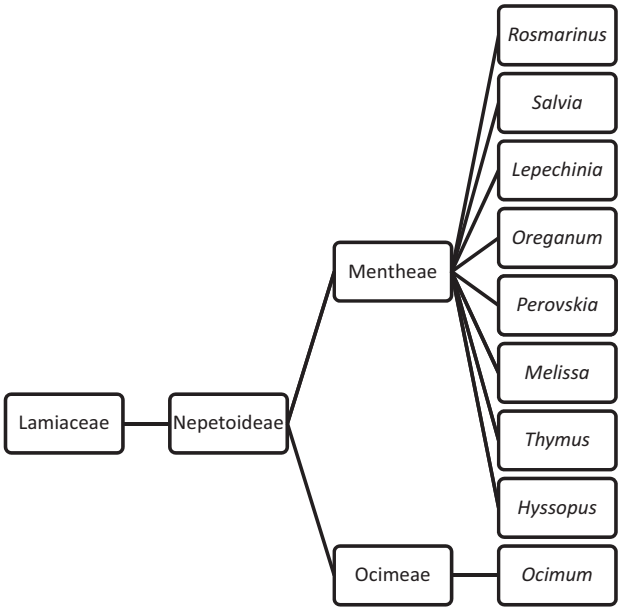

Fig. 2. Occurrence of carnosic acid (**1**) in the plant kingdom, exclusively in the Lamiaceae.

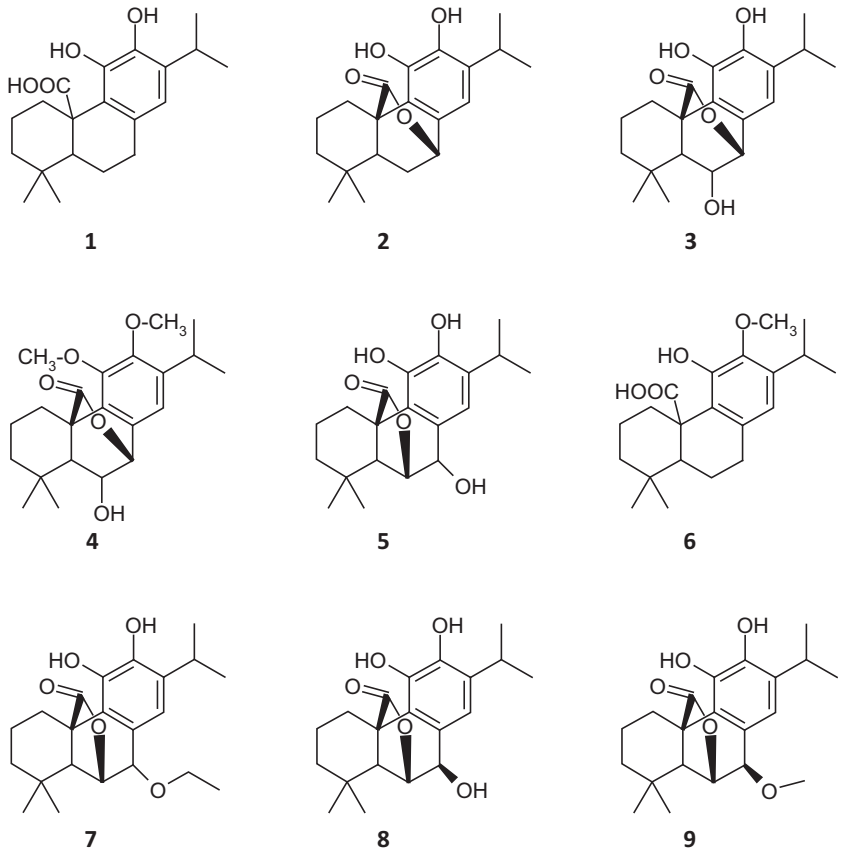

Fig. 1. Structures of carnosic acid (**1**) and of phenolic diterpenes similar to carnosic acid (**1**): **1** carnosic acid; **2** carnosol; **3** isorosmanol; **4** 11,12-di-*O*-methylisorosmanol; **5** rosmanol; **6** 12-*O*-methylcarnosic acid; **7** rosmanol-9-ethyl ether; **8** epirosmanol; **9** 7-methyl-epirosmanol.

(Abreu et al., 2008), whereas in *R. officinalis*, content ranges from 3 to 50 mg g^{-1} DW (Richheimer et al., 1996; Schwarz and Ternes, 1992; Wenkert et al., 1965) depending upon variety, developmental stage and environmental conditions (Munné-Bosch and Alegre, 2000). Why is carnosic acid (**1**) so poorly distributed across the plant kingdom? It is present in only one plant family, in only one (Nepetoideae) out of 10 Lamiaceae subfamilies, in only two (Mentheae, Ocimum) out of 66 tribes, and in only nine out of 220 genera (Fig. 2). Such a small proportion (4%) of Lamiaceae genera containing carnosic acid (**1**) probably results from a very specific evolutionary strategy but also from a paucity of research, as a great proportion of plant species still remain unknown and/or un-investigated. Thus, although carnosic acid (**1**) seems to be exclusive to the Lamiaceae family, it belongs to abietane diterpenes, together with ferruginol and abietic acid, which appeared early in evolutionary history and are found in resins from gymnosperms (Otto et al., 2002). Its notable distinction is in having two hydroxyl groups in the *ortho* positions at C_{11} and C_{12}, which greatly contribute to its antioxidative properties (Fig. 1).

Carnosic acid (**1**) is not distributed evenly in plants, being mostly present in the aerial parts, a hint of its proposed role *in planta* (see below). In rosemary, it was mainly found in photosynthetic tissues: leaves, sepals and petals, with leaves the most abundant (10–15 mg g^{-1} FW), followed by sepals (5–10 mg g^{-1} FW) and petals (<2 mg g^{-1} FW) (del Bano et al., 2003; Luis and Johnson, 2005). Carnosic acid (**1**) levels also depend on the developmental stage. Within 15 days of growth carnosic acid (**1**) increased by three folds in leaves of three-month old plants, then decreased as rapidly down to initial levels and remained at constant levels for months (del Bano et al., 2003). In *S. officinalis* leaves, carnosic acid (**1**) and carnosol (**2**) levels increased with senescence (Abreu et al., 2008). Environmental conditions also have a great impact on carnosic acid (**1**) abundance. Its concentrations decreased at high temperatures and low precipitation rates (Luis and Johnson, 2005) in parallel with dropping relative water content (RWC) in the plant (Munné-Bosch and Alegre, 2000; Munné-Bosch and Alegre, 2003). Nutrient availability was shown to significantly influence carnosic acid (**1**) abundance. Salt stress generated by high levels of Na^+ reduced carnosic acid (**1**) content in *S. officinalis* leaves while addition of K^+ and Ca^{2+} led to accumulation of carnosic acid *S. officinalis* leaves (Tounekti et al., 2010, 2011).

On a cell-specific level, isolated rosemary leaf trichomes or solvent after leaf dipping that probably mostly contained trichome compounds, appeared to accumulate carnosic acid (**1**) more than other leaf tissues (Brückner et al., 2014). Authors report that while both carnosic acid (**1**) and carnosol (**2**) accumulated inside leaves, isolated trichomes primarily contained carnosic acid (**1**). After removal of trichomes, the remaining leaf material contained relatively more carnosol (by ~40%) than carnosic acid. On the other hand, the removed trichomes contained mainly carnosic acid with relatively low amounts of carnosol. Unfortunately, authors do not report on quantities of carnosic acid and carnosol per trichome weight, or per leaf (without trichomes) weight. Such data would have informed on the trichome contribution to carnosic acid levels in the overall leaf material. However, authors do report that carnosic acid was more abundant than carnosol in both young and old rosemary leaves and that its levels decreased by ~40% with ageing of the Alderney variety (Brückner et al., 2014). As carnosol (**2**) is relatively more abundant in leaves without trichomes but carnosic acid (**1**) is more abundant in leaves with trichomes, the overall findings therefore imply that important part of carnosic acid (**1**) in rosemary leaves would be localized in trichomes. However, this is difficult to conclude as the data are not reported per weight. Brückner et al. (2014) report rather high carnosol/carnosic acid ratio as % carnosol based on the calculation [carnosol/(carnosic acid + carnosol)] increased from ~38% in young leaves to ~45% in old leaves (calculated from data published by Brückner et al. (2014)). Similar high (~42%) % carnosol (reported to (carnosic acid + carnosol)) were found in rosemary leaves subjected to drought and high light (Munné-Bosch and Alegre, 2000). Munné-Bosch and Alegre (2000) data on carnosol (**2**) content in rosemary leaves contradict Abreu et al. (2008) findings reported for senescing *S. officinalis* leaves. Abreu et al. (2008) have shown that carnosic acid (**1**) and carnosol (**2**) levels increased with senescence and the same % carnosol based on the same calculation [carnosol/(carnosic acid + carnosol)] was found in the whole *Salvia* leaf (Abreu et al., 2008) as in rosemary trichomes (Brückner et al., 2014), which approximated to 15–16% (Table 1). Our data obtained using freeze-dried and ground non-stressed leaves that would correspond to 'old' leaves according to Brückner et al. (2014), from ~40 rosemary varieties, including Alderney, show that % carnosol based on the same calculation [carnosol/(carnosic acid + carnosol)] approximated to 6% (Table 1). Our findings rather corroborate those reported in non-senescent *S. officinalis* leaves (Abreu et al., 2008) (Table 1). It must be noted that our aged plants were not grown in stress conditions. Thus, it remains unclear whether it is ageing, oxidative stress or most likely ageing in oxidative stress conditions, that impacts the ratio of carnosic acid and of carnosol in the whole leaf (with trichomes). It also must be highlighted that phenolic diterpenes from whole leaves and from trichomes alone were extracted using different procedures and notably different organic solvents; moreover trichome isolation (Brückner et al., 2014) may have induced oxidative stress in the rest of leaves and yield in an increase in carnosol levels. Nevertheless, Brückner et al. (2014) have shown that both *RoCPS1* and *RoKSL2* genes are rather expressed in glandular trichomes than in leaves which points at glandular trichomes as the main organs that would contribute, at least to the first steps, of the biosynthetic pathway to carnosic acid. Once biosynthesised in trichomes, carnosic acid precursors or carnosic acid itself, may be transported to other leaf cells and then converted into carnosic acid or into its derivatives, respectively.

Subcellular localisation studies have so far revealed that carnosic acid (**1**) is localised in the plastids, although its exact site remains to be determined. (Munné-Bosch and Alegre, 2001) have detected ~6 times more carnosic acid (**1**) in rosemary leaf chloroplasts as compared to the overall leaf, indicating that carnosic acid

Table 1
Percentage in carnosol (**2**) expressed as ([carnosol])/([carnosic acid] + [carnosol]) in *Rosmarinus* and *Salvia* leaves upon ageing, senescence or (oxidative) stress.

Species	Variety	Treatment	[Carnosol/(carnosol + carnosic acid)] (%)	References
Rosmarinus officinalis	Alderney	Young	38	Brückner et al. (2014)
Rosmarinus officinalis	Alderney	Aged	45	Brückner et al. (2014)
Rosmarinus officinalis		Aged and stressed	42	Munné-Bosch and Alegre (2000)
Rosmarinus officinalis		Aged and non stressed	36	Munné-Bosch and Alegre (2000)
Rosmarinus officinalis	Alderney	Aged non stressed	6	This study
Salvia officinalis		Non senescening	7	Abreu et al. (2008)
Salvia officinalis		Early senescening	9	Abreu et al. (2008)
Salvia officinalis		Late senescening	16	Abreu et al. (2008)

is rather concentrated in chloroplasts. Carnosic acid was not detected in the endoplasmic reticulum, the Golgi apparatus nor in the plasma membrane (Munné-Bosch and Alegre, 2001). It is not known whether carnosic acid (**1**) occurs in other organelles, including the vacuole. However, given its lipophilic feature, it is reasonable to suggest that if present in other organelles, it would rather localise to their lipophilic parts. Further research is required to address the ultra-cellular distribution of carnosic acid (**1**) not only in plastids but also in other, so far not studied, organelles.

3. Biosynthesis of carnosic acid

The biosynthesis of carnosic acid (**1**) has not entirely been unravelled. Investigations have mainly focused on general terpenoid biosynthesis, with specific studies regarding diterpenes in *Salvia*, whereas rosemary has rarely received any attention. Carnosic acid (**1**) biosynthesis probably follows the 'biogenetic isoprene rule' (Ruzicka, 1953) and it can be reasonably presumed that the construction of carnosic acid (**1**) would involve enzyme-assisted electrophilic elongation, cyclisation and rearrangements of the basic skeleton.

Carnosic acid (**1**) belongs to the labdane-related class of diterpenoids, whose biosynthesis is most probably initiated by a sequential pair of cyclisation reactions. Due to the plastidial localisation of diterpene synthases, plant diterpenoids normally originate from the plastidic 1-deoxyxylulose-5-phosphate (DXP) pathway. However, a contribution of the cytosolic mevalonate (MVA) pathway is not to be excluded, given the significant number of studies showing the existence of cross-talk between the two pathways (Bick and Lange, 2003; Yang et al., 2012).

Although no biosynthesis pathway for carnosic acid of rosemary has yet been proposed, Brückner et al. (2014) suggest that one reasonable intermediate is abietatriene, tricyclic abietane diterpene with an aromatized C-ring and molecular mass of 270. Indeed, the typical catechol group would be produced by double hydroxylation of abietatriene on the C-ring. Multiple oxidations on the 20-carbon backbone of the diterpenoid would provide the carboxyl group present in carnosic acid. Brückner et al. (2014) also suggest that since the cyclisation of GGDP by olefin-producing diterpene synthases typically results in products with a parent mass of 272 rather than 270, it seems unreasonable to think that abietatriene would be a direct product of such enzymes. Authors suggest that biosynthetic pathway en route to carnosic acid would involve the transformation of GGDP into copalyl diphosphate (CDP), which would convert into miltiradiene. Miltiradiene contains a cyclohexa-1,4-diene moiety that imposes a planar configuration on the distal ring, which is ideally placed for aromatisation, as required for the production of carnosic acid (**1**).

Studies conducted in *Salvia* or in Gymnosperms (Gao et al., 2009; Peters, 2010) show that the characteristic fused bicyclic hydrocarbon structure of labdane-like diterpenes is formed from the diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP) in an initial carbon–carbon double bond protonation-initiated reaction catalysed by class II diterpene cyclases. These typically form labdadienyl/copalyl diphosphate (CDP) with corresponding enzymes CDP synthases (CPS). This initial cyclisation by protonation involves a DxDD motif located at the interface of the β and γ structural domain of the enzyme (Cao et al., 2010; Köksal et al., 2011). At this step, the CDP initial stereochemistry is established. For instance, normal-CDP isomer is a precursor in the biosynthesis of similar *Salvia* labdane diterpenes, tanshinones (Gao et al., 2009; Peters, 2010) (Fig. 3).

As also shown in *Salvia* roots, additional stereocentres are generated through the formation of a carbocation in the subsequent cyclisation by ionisation of the diphosphate linkage of CDP. This reaction is catalysed by class I terpene synthases, including kaurene synthase-like (KSL) (Gao et al., 2009; Peters, 2010) and involves a DDxxD motif in the α domain of the enzyme (Cao et al., 2010; Köksal et al., 2011). At this stage, CDP can be transformed into a variety of polycyclic diterpenoids, such as carnosic acid. The above findings reported in *Salvia* have recently been confirmed in rosemary (Brückner et al., 2014) who have shown that RoCPS1 in combination with either RoKSL1 or RoKSL2 produce miltiradiene from GGDP (Fig. 3). They have functionally characterised RoCPS1 and RoKSLs and reported these enzymes as novel CPS producing normal CDP, and novel KSL enzymes, acting as miltiradiene synthases. Moreover, RoKSLs (Brückner et al., 2014) and SmKSL share high sequence similarity. This finding combined with the same product profile suggests that RoKSLs together with SmKSL may share the same substrate (normal CDP) specificity (Brückner et al., 2014) (Fig. 3).

For further steps in carnosic acid biosynthesis, it is reasonable to base on the biosynthesis of tanshinones in *Salvia* roots (Guo et al., 2013). It is reasonable to suggest that miltiradiene in rosemary, as in *Salvia* may undergo similar transformations to yield carnosic acid (Fig. 3). Guo et al. (2013) have shown *in vitro* and *in vivo* that CYP76AH1 enzyme catalyses a unique four-electron oxidation cascade on miltiradiene to produce ferruginol. Zi and Peters (2013) reported that the rosemary CYP ortholog CYP76AH4 hydroxylates the oxidised aromatic intermediate abietatriene into ferruginol and that miltiradiene can spontaneously oxidise into abietatriene (Fig. 3). By expressing *SmKSL*, or by co-expressing *RoC-*

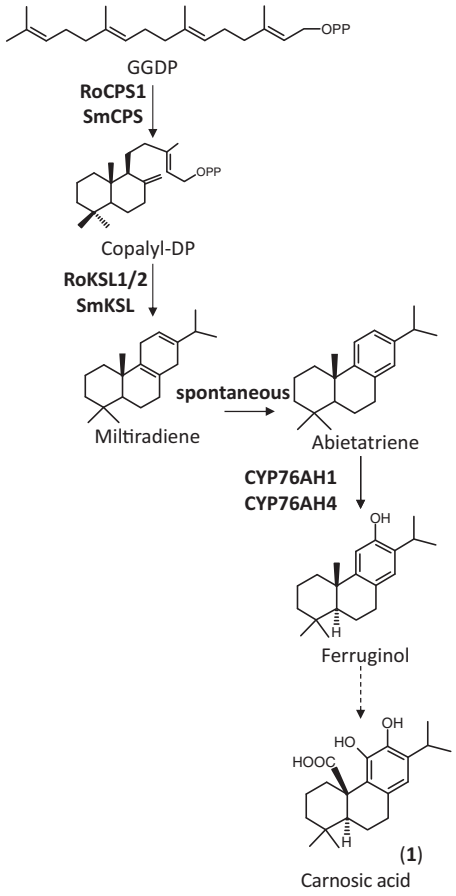

Fig. 3. Proposed biosynthetic pathway of carnosic acid based on findings reported by Brückner et al. (2014), Gao et al. (2009) and Guo et al. (2013). GGPP: geranylgeranyl diphosphate; CPS: copalyl diphosphate synthase; KSL: kaurene synthase-like; solid arrows indicate the established relationships, and dashed arrows indicate hypothetical relationships.

PS1 and *RoKSL*, in *Nicotiana benthamiana* leaves, Brückner et al. (2014) detected both miltiradiene and abietatriene. The latter compounds were also recovered in leaf exudates of rosemary plants (Brückner et al., 2014). Yet, *in vitro* assays with SmKSL lead to the recovery of only miltiradiene (Gao et al., 2009). These findings imply that abietatriene is formed *in planta* by a spontaneous oxidation rather than by the enzymatic activity of KSL. Interestingly (Guo et al., 2013) do not report on the detection of abietatriene intermediate in *Salvia miltiorrhiza* hairy roots fed with ^{13}C-labeled miltiradiene, however Zi and Peters (2013) report that abietatriene is also a precursor of CYP76AH1. (Guo et al., 2013) report on close homology between several *R. officinalis* isotigs and *S. miltiorrhiza* CYP76AH1 (~85% nucleotide sequence identity). Findings of (Guo et al., 2013) and of (Zi and Peters, 2013), and the presence of ferruginol in Lamiaceae (Yang et al., 1996) capable of carnosic acid (**1**) biosynthesis, suggest that ferruginol is a potential intermediate in carnosic acid (**1**) biosynthesis (Fig. 3).

Studies reported on the biosynthesis of labdane-like diterpenes in *S. miltiorrhiza* and in *R. officinalis* were conducted in roots and in leaves, respectively. Thus, plants from the two Lamiaceae genera seem to share at least initial steps of biosynthetic pathways en route to labdane-like diterpenes, be it in roots or in leaves. Close homology between *Salvia* and *Rosmarinus* isotigs reported for CYP enzymes as well as high homologies between SmKSL and RoKSLs enzymes, add to the morphological similarities between the two genera. They both have two instead of the usual four stamens encountered in the mint family. This reinforces a long-lasting question whether *Rosmarinus* should remain in a separate genus from *Salvia*.

It is evident that our knowledge of the biosynthesis of carnosic acid (**1**) in *Salvia* or in *Rosmarinus* can be described, at best, as rudimentary. Labelling experiments and further enzyme characterization are required to confirm the hypothetical scheme put forward, which is largely based in evidence obtained for the biosynthesis of comparable compounds in other species.

Although the biosynthetic pathway of carnosic acid (**1**) in rosemary has received even less attention than labdane-like phenolic terpene biosynthesis in *Salvia*, compounds involved in labdane-like phenolic terpene biosynthesis in *Salvia* were identified in *R. officinalis* (Brückner et al., 2014; Zi and Peters, 2013) indicating probable similarities between the pathways in these two genera.

4. What evidence is there that carnosic acid is an antioxidant?

Carnosic acid (**1**) plus carnosol (**2**) have been suggested to account for over 90% of the antioxidant properties of rosemary extract (Aruoma et al., 1992), although this has not yet been systematically verified. This high contribution of carnosic acid (**1**) to antioxidative response of rosemary extract is probably also attributed to the great abundance of carnosic acid as compared to other rosemary phenolic diterpenes. It has been proposed that the radical scavenging activity of carnosic acid (**1**) follows a mechanism analogous to that of other antioxidants, including α-tocopherol, and is caused by the presence of the two *O*-phenolic hydroxyl groups found at C_{11} and C_{12} (catechol moiety) (Richhelmer et al., 1999). Upon oxidation, carnosic acid (**1**) and α-tocopherol display different antioxidative capacities that depend upon the lipid composition of the matrix and more so upon oxidative conditions (Hopia et al., 1996; Huang et al., 1996). In emulsions, at 37 °C α-tocopherol better preserves lipids from oxidation than carnosic acid (**1**) (Hopia et al., 1996). At higher temperatures (60 °C), α-tocopherol is not as efficient as carnosic acid (**1**) in protecting lipids from oxidation (Huang et al., 1996). Yet at higher temperatures (60 °C) carnosic acid (**1**) is consumed faster than α-tocopherol, indicating that it is the oxidation products of carnosic acid (**1**) that contribute to the greater antioxidative response. Moreover, it is methyl carnosoate, rather than carnosic acid (**1**) or α-tocopherol, that is the most active antioxidant in w/o emulsions but it is less active than Trolox in o/w emulsions (Schwarz et al., 2000).

Although carnosic acid (**1**) is considered to be unstable, particularly in solvents, it seems to have an increased stability in food matrices. Considerable amounts of carnosic acid (**1**) remain present following the ripening period of raw sausage. In lyophilized chicken meat, carnosic acid (**1**) degrades slowly and decreases storage dependent degradation of α- and γ-tocopherol (Ternes and Schwarz, 1995). Moreover, it is noteworthy that the recovery efficiencies for carnosic acid (**1**) range from 68.1% to 96.2% depending on the food matrices (Ternes and Schwarz, 1995). Besides food matrices, the stability and therefore the ability to prevent oxidation of carnosic acid (**1**) depends upon oxidative conditions. Schwarz et al. (1992) have shown that carnosic acid (**1**) degraded less as compared to other phenolic diterpenes, in different conditions involving an increase in temperature. This indication on carnosic acid (**1**) resistance to heat implies its superior ability to protect from oxidation. Carnosic acid (**1**) has been reported to exert greater antioxidative activity than the widely-used, synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Cuvelier et al., 1994; Erkan et al., 2009; Luis and Johnson, 2005) in sunflower oil or in soybean oil (Richheimer et al., 1996), or than tocopherols in bulk corn oil (Cuvelier et al., 1994; Hopia et al., 1996; Huang et al., 1996). It was shown to more efficiently decrease the formation of hydroperoxides of certain fatty acids (Erkan et al., 2009; Hopia et al., 1996). Such *in vitro* studies, based on unspecific free radical assays, mainly provoked by an increase in temperature in dark conditions, point at carnosic acid (**1**) as a possible scavenger of reactive oxygen species (ROS). Because of this lack in specificity, these studies could not show differences between antioxidative activities of carnosic acid (**1**) against distinct ROS (singlet oxygen ($^1O_2$), H_2O_2, superoxide). On the other hand, scavenging capacities of carnosic acid (**1**) mixed with other compounds within rosemary extracts, against specific ROS have been reported (Wada et al., 2004). However, because of the presence of other compounds in rosemary extracts, these studies do not reveal at which extent carnosic acid (**1**) itself contributes to the antioxidative response to distinct ROS. Future studies should address such questions. Thus, once again, for a molecule of such potential value, the lack of sound evidence for its mode of action is surprising.

5. Antioxidant role *in planta* of carnosic acid

Considering its tissue and so far identified organellar distribution, it is probable that carnosic acid (**1**) acts to protect chloroplasts and chloroplastic and maybe other organelle membranes from oxidative stress. Upon drought (Munné-Bosch and Alegre, 2003) and leaf photooxidation, it may scavenge reactive oxygen species (ROS) giving rise to diterpene alcohols, mainly isorosmanol (**3**) and oxidation products including *O*-methylated compounds (Fig. 1). As methylations increase compound lipophilicity they probably facilitate the removal of ROS from membranes. Such processes result in the production of *O*-methylated diterpenes, including 11,12-di-*O*-methylisorosmanol (**4**), 11,12-di-*O*-methylrosmanol and 12-*O*-methylcarnosic acid (**6**), which are found at highest levels in plasma membrane, rather than in chloroplasts, the Golgi apparatus or the endoplasmic reticulum (Luis et al., 1994; Munné-Bosch and Alegre, 2001). Although *O*-methylation of diterpenes considerably decreases their antioxidant activity by disrupting the catechol moiety, the remaining hydroxyl group may form hydrogen bonds with the head group of a phospholipid and its dimensions may allow cooperative van der Waals attractive

forces to reinforce and stabilise the lipid chain and therefore the stability of the plasma membrane (Havaux, 1998; Munné-Bosch and Alegre, 2001).

Salvia and *Rosmarinus* species grow in high light conditions and their photosynthetic tissues suffer from photooxidation (Munné-Bosch and Alegre, 2001). Triantaphylides et al. (2008) have shown that oxidative damage provoked by high light specifically involves singlet oxygen ($^1O_2$) as the major ROS induced by the excess energy in photosynthetic membranes (Triantaphylides et al., 2008). Plants have evolved several isoprenoids involved in mechanisms to dissipate this excess energy. Carotenoids and tocopherols most efficiently protect photosynthetic lipids from photooxidation as they are capable of ROS scavenging. During this process, they particularly preserve from $^1O_2$ oxidation (Fukuzawa et al., 1998) and dissipate the excess energy as heat (DellaPenna, 1999). Lipophilic rosemary extracts containing an array of compounds, including carnosic acid, displayed ROS and, notably, $^1O_2$, scavenging properties (Wada et al., 2004). Nevertheless, it is still not known to what extent carnosic acid (**1**) may protect lipids from particular ROS. Both *in vitro* and *in planta* studies are required to define the capacities of carnosic acid (**1**) to protect lipids from different types of ROS. Further studies should also identify which oxidation products are specifically formed from carnosic acid (**1**) and whether these differ for distinct ROS.

Carotenoids and tocopherols appeared early in evolutionary history and are present in all photosynthetic organisms. In contrast, carnosic acid (**1**) has evolved only within the Lamiaceae where, like tocopherols and carotenoids, it is located in chloroplasts. Moreover, carnosic acid-containing species are significantly less abundant in tocopherols (Abreu et al., 2008), possibly because carnosic acid (**1**) is taking over their antioxidative role. It can even be proposed that, upon (photo)oxidation, carnosic acid (**1**) stabilises, protects and cooperates with tocopherols and carotenoids as an additional mechanism for photoprotection (Peñuelas and Munné-Bosch, 2005). While this suggestion remains to be tested, it would appear that some Lamiaceae, including *Rosmarinus* and some *Salvia* species, may have evolved three rather than two types of lipophilic antioxidants to insure the protection of chloroplasts from oxidation in response to harsh environmental conditions (Peñuelas and Munné-Bosch, 2005). The suggestion related to photooxidative stress is in line with Munné-Bosch and Alegre (2003) contention referring to chloroplasts of water stressed plants where it was suggested that carnosic acid (**1**) in combination with α-tocopherol and ascorbate helped to prevent oxidative damage in functionally interdependent manner.

Based on the surprisingly small body of evidence in the literature, carnosic acid (**1**) appears to be the most abundant lipophilic antioxidant compound in rosemary leaves. Such tremendous abundance may contribute to its high antioxidative extent. However, it is clear that further studies are required to confirm this and to determine its mode(s) of action.

6. Is carnosic acid also an antimicrobial compound?

Rosemary extracts rich in carnosic acid (**1**) have been shown to have antimicrobial activity (Campo and Amiot, 2000) and studies on various pathogens have been performed to determine which rosemary compounds were responsible for this activity.

The impact of carnosic acid (**1**) was assessed by evaluating the antilisterial effect of two rosemary extracts containing 20 or 40% carnosic acid. Both showed an antimicrobial activity but the minimal inhibitory concentration (MIC) value of the later was two times lower than the former, suggesting that carnosic acid (**1**) was the most active compound (Romano et al., 2009). This result was confirmed by determining the antimicrobial activity of 8 major rosemary phenolics. Carnosic acid (**1**) was thus shown to have the strongest antilisterial activity (Campo et al., 2003) when tested for 24 h at 30 °C after inoculation. This antilisterial activity was further improved at low pH and high NaCl content. Other Gram positive bacteria, such as Bacillus, Enterococcus, Streptococcus or Staphylococcus, or Gram negative bacteria, such as Escherichia, Salmonella or Campylobacter, were also responsive to carnosic acid (**1**) (Bernardes et al., 2010; Horiuchi et al., 2007; Moreno et al., 2006; Rozman and Jersek, 2009; Shayma'a et al., 2011; Klančnik et al., 2009, 2010). Even if most studies showed only a moderate effect on Gram positive and an even less intense effect on Gram negative bacteria, synergisms may occur with other antimicrobials. It was shown for instance on Tetracycline, Erythromycin or Ethidium Bromide which antimicrobial actions were improved up to 8-fold against *Staphylococcus aureus* by addition of 10 μg/mL carnosic acid (**1**) (Oluwatuyi et al., 2004) and up to 16-fold against *Enterococcus faecium* and *Enterococcus faecalis* by addition of 8 μg/mL carnosic acid (**1**) (Horiuchi et al., 2007). Mechanisms involved in antimicrobial activities of phenolic diterpenes have not yet been elucidated, but it has been suggested that the lipophilic structure of these compounds allows them to insert into the bacterial membrane (Cowan, 1999), where their hydrogen-bond-donor group(s) could interact with membrane phosphorylated groups (Souza et al., 2011) or that carnosic acid acts as a modulator of the ethidium bromide efflux which is responsible for membrane permeability (Ojeda-Sana et al., 2013).

7. Exploitation of carnosic acid in the food, nutrition and health, and cosmetics industries

7.1. Carnosic acid and carnosol – as the main active antioxidants of food additives in Europe (E392)

Rosemary extracts have been used as antioxidants by the food industry for more than 20 years. The first rosemary extracts used as antioxidants in foods were ingredients derived from oil-soluble, flavourful rosemary oleoresins produced by solvent extraction. Such rosemary extracts were mostly used in savoury applications and labelled as flavours, although primarily used for food preservation purposes.

Identification of carnosic acid (**1**) as probably the key contributor to the antioxidant activity was an important milestone in the history of the use of rosemary extracts (see Section 7.2). It is considered as a qualitative tracer that allows standardising the antioxidant potency. As deodorised rosemary extracts with an assured content in carnosic acid (**1**) have become increasingly available since the 1990s, their use for protection from oxidation of a large variety of food matrices significantly increased.

In recognition of their efficiency and upon the request of the food industry, in 2010 rosemary extracts were classified as food additives by the European Commission and assigned the number E392 (Commission Directives 2010/67/EU and 2010/69/EU repealed in 2013 by EU regulation 231/2012 and 1333/2008). "Antioxidant: extracts of rosemary" are to be produced with one of the four extraction processes described in the regulation, by means of solvent extraction (ethanol, acetone or ethanol followed by hexane) or supercritical carbon dioxide extraction, paying respect to purity criteria. According to the EU regulation, only deodorised rosemary extracts containing carnosic acid (**1**) and carnosol (**2**) are considered additives. Indeed, carnosic acid (**1**) and its derivative carnosol (**2**) are listed as key antioxidant compounds in rosemary extracts and E392 dosage limitations are expressed as levels of carnosic acid (**1**) and carnosol (**2**), rather than of the whole rosemary extract. Application areas are food matrices, including oils, animal fats, sauces, bakery wares, meat and fish products etc.

The EU regulation also established a criterion based on the ratio of reference antioxidant compounds (carnosic acid (**1**) and carnosol (**2**)) to reference key volatile compounds (main flavouring constituents of rosemary essential oils: borneol, bornyl acetate, camphor, 1,8-cineol, verbenone). It thus gives information on the level of deodorisation of rosemary extracts and on their antioxidative capacities. Antioxidant rosemary extracts must have a content in reference antioxidant compounds that is at least 15 times higher than their content in key volatiles compounds:

$$\frac{\text{Total \% w/w of carnosic acid and carnosol}}{\text{Total \% w/w of reference key volatiles}} \geqslant 15$$

Nevertheless, certain types of non-deodorised rosemary extracts, including rosemary essential oils or rosemary oleoresins, not standardised to antioxidant active compounds (carnosic acid (**1**) and carnosol (**2**)), are still used in the food industry for flavouring purposes only. It would not have made sense to include these types of extracts in the scope of the definition of the additive as the regulation aimed at covering antioxidant rosemary extracts only.

The European Union is not the only area where rosemary extracts are approved as food additives. In Japan, rosemary extracts are listed under number 365 in the List of Existing Additives and defined as "a substance composed mainly of carnosic acid, carnosol (**2**) and rosmanol (**5**) obtained from rosemary leaves or flowers". Chinese food additive regulation GB2760-2011 approves the use of rosemary extracts under the Chinese numbering system CNS 04.017. Interestingly, it is a carnosic acid (**1**) derivative, carnosol (**2**) that is listed as the reference antioxidant compound in oil-soluble extracts. In most other countries, including the USA, rosemary extracts do not yet have any official status as technological additive.

7.2. Carnosic acid in nutrition and health, and cosmetics

Antioxidative properties of carnosic acid (**1**) have been translated into photoprotective activity against UVA oxidation in human dermal fibroblasts, which could indicate a potential for dermal application (Offord et al., 2002). Numerous health applications based on antioxidative features of carnosic acid (**1**) and carnosol (**2**) (Bruno et al., 1991), including reduction of cytochrome *c* and protection of the protein α_1-antiproteinase against inactivation (Aruoma et al., 1992) have been developed. Anti-carcinogenic properties were assigned to carnosic acid (**1**) (Danilenko and Studzinski, 2004; Einbond et al., 2012; Rajasekaran et al., 2013; Tsai et al., 2011) extracted mainly from *R. officinalis* (Barni et al., 2012; Costa et al., 2007; Dickmann et al., 2012; Einbond et al., 2012; Lee et al., 2007; Lopez-Jimenez et al., 2013; Nakamura et al., 2006; Ngo et al., 2011; Sharabani et al., 2006; Steiner et al., 2001; Tsai et al., 2011; Yesil-Celiktas et al., 2010), but also from *Salvia* (Bauer et al., 2012; Guerrero et al., 2006; Hussein et al., 2007; Kontogianni et al., 2013; Masuda et al., 2002; Rau et al., 2006b), *Ocimum* (Baliga et al., 2013) or *Perovskia* (Aoyagi et al., 2006). Plant extracts abundant in carnosic acid (**1**) were found to be anti-carcinogenic due to their proapoptotic (Tsai et al., 2011), antiproliferative (Kontogianni et al., 2013), anti-angiogenic (Lopez-Jimenez et al., 2013), chemoprotective (Costa et al., 2007), antitumour (Aoyagi et al., 2006; Sharabani et al., 2006) or antiplatelet (Lee et al., 2007) properties. In a number of *in vitro* studies, plant extracts containing carnosic acid (**1**) showed preventive (Nabekura et al., 2010; Ngo et al., 2011) and inhibitory (Barni et al., 2012; Bauer et al., 2012; Dickmann et al., 2012; Nabekura et al., 2010; Steiner et al., 2001; Yesil-Celiktas et al., 2010) properties against cancer. Numerous other investigations have shown that plant extracts rich in carnosic acid (**1**) exhibit anti-inflammatory features, applied in health (Bauer et al., 2012; Hadad and Levy, 2012; Kamatou et al., 2009; Kuhlmann and Roehl, 2006; Kuo et al., 2011; Mengoni et al., 2011; Molnar and Garai, 2005; Oh et al., 2012; Poeckel et al., 2008; Rajasekaran et al., 2013; Yanagitai et al., 2012; Yu et al., 2009) or cosmetics, as it suppresses interleukins, blocks nitric oxide release and the proto-oncogene tyrosine-protein kinase Src pathway (Oh et al., 2012). Plant extracts containing carnosic acid (**1**) were also reported to have anti-adipogenic properties enabling weight loss, and proved efficient in the treatment of hyperglycaemia (Dickmann et al., 2012; Ibarra et al., 2011; Rau et al., 2006a,b; Tashmukhamedova et al., 1988; Wang et al., 2012). Other studies aimed at developing applications have shown an anti-ageing potential of rosemary extract containing carnosic acid (**1**) by preventing oxidative alteration of skin surface lipids or by the biophylaxis mechanism (Calabrese et al., 2000; Kosaka, 2012). Oral health care is another cosmetics-related application of carnosic acid: *in vitro* studies have shown that it efficiently protected against typical cariogenic microorganisms (Bernardes et al., 2010).

Aforementioned activities, i.e. antioxidant, anti-inflammatory, body weight reduction…, were validated by several *in vivo* studies (Table 2). Carnosic acid (**1**) bioavailability was shown by Doolaege et al. (2011) and Jordán et al. (2014), who reported that carnosic acid (**1**) was assimilated in muscular tissue and remained in the circulatory system for several hours (Romo Vaquero et al., 2013).

8. Phenolic diterpenes similar to carnosic acid

Phenolic diterpenes similar in structure to carnosic acid (**1**) have been isolated from both *Salvia* and *Rosmarinus* species. Such compounds include carnosol (**2**) (Brieskorn et al., 1964; Brieskorn and Fuchs, 1962), rosmanol (**5**) (Nakatani and Inatani, 1984), and rosmaridiphenol (Houlihan et al., 1984), the principal degradation products of carnosic acid. Minor degradation derivatives of carnosic acid (**1**) are epirosmanol (**8**) (Cuvelier et al., 1994), 7-methyl-epirosmanol (**9**) (Schwarz and Ternes, 1992) and probably rosmanol 9-ethyl ether (Djarmati et al., 1991). Very early on, Wenkert et al. (1965) demonstrated that carnosol (**2**) (pikrosalvin) is an oxidative 'artifact' (derivative) of carnosic acid. Its formation takes place in the presence of oxygen both after leaf harvest and in the leaves upon drying. (Note that carnosol (**2**) also exerts antioxidative effects (see Section 4)) (Wenkert et al., 1965). Other authors have also suggested that rosmanol (**5**) and rosmaridiphenol are generated from carnosic acid (**1**) during extraction processes. They have even speculated that fresh rosemary leaves do not contain carnosol (**2**), which would leave carnosic acid (**1**) as the only phenolic diterpene present in the native state in rosemary and sage. Luis et al. (1994) have shown, however, that carnosic acid (**1**) can give rise to highly oxidised diterpenes including rosmanol (**5**), isorosmanol (**3**) or 11,12-di-*O*-methylrosmanol and 11,12-di-*O*-methylcarnosol *in planta*. This conversion would involve enzymatic dehydrogenation processes and the participation of singlet oxygen (Luis et al., 1994). During such processes, the methylation of the *O*-phenolic hydroxyl groups eliminates the radical scavenging activity of the molecule and increases its lipid solubility (Brieskorn and Dumling, 1969). The presence of diterpenes similar in structure to carnosic acid (**1**), including carnosol (**2**), rosmanol (**5**), isorosmanol (**3**), methylated compounds, was confirmed in plants subjected to high light and drought (Munné-Bosch and Alegre, 2000; Munné-Bosch et al., 1999; Munné-Bosch and Alegre, 2001).

While carnosic acid (**1**) could be found in plastids even upon stress, methylated compounds similar in structure to carnosic acid (**1**) were found in chloroplasts, endoplasmic reticulum, Golgi apparatus and in plasma membrane (Munné-Bosch and Alegre, 2001). Again, it is not known whether these compounds occur in other organelles. While levels in carnosic acid (**1**), carnosol (**2**) and in methylated compounds similar to carnosic acid (**1**) decrease in

Table 2
Carnosic acid uses in food, *in vivo* and in clinical studies.

#	# subjects	Time	Protocol	Results	Effect	References
1	32 hamsters	11 weeks	Administration of groups: I: 0.5% DMBA (n = 12); II: 0.5% DMBA + potassium apigenin (n = 8); III: 0.5% DMBA + carnosic acid (n = 12)	With CA, only one malignancy was recorded, showing the smallest volume of all the recorded tumour lesions.	Chemoprotective	Gómez-García et al. (2013)
2	40 mice	12 weeks	Administration of high-fat diet, without CA or supplemented with 0.01% (w/w) CA or 0.02% (w/w) CA	CA suppressed HFD-induced hepatic steatosis and fatty liver-related metabolic disorders	Hepatic steatosis suppressant	Park and Mun (2013)
3	42 rats	–	Administration of 1 mL of CA solution (CA: 10 mg/kg) intraperitoneally, 1 h prior to surgery	CA could significantly improve short-term spatial and learning memory scores following their impairment by Aβ toxicity	Chemopreventive against neurodegenerative disorders	Rasoolijazi et al. (2013)
4	24 rats	64 days	Administration of diet supplemented with 0.5% w/w rosemary extract (40% CA)	CA modified microbiota composition and decreases β-glucosidase activity in the caecum of rats while it increased fibre faecal elimination	Body weight gain reduction	Romo-Vaquero et al. (2014)
5	12 rats	14 days	Administration of diet supplemented with 50 mg rosemary extract (3 mg CA) daily	Rosemary extract reduced free-radical-induced lipid peroxidation *in vivo*	Antioxidant	Kuzmenko et al. (1999)
6	40 healthy volunteers	–	Double-blind placebo-controlled study treated with 2% sage extract (rich in carnosol & CA)	Sage extract significantly reduced the ultraviolet-induced erythema, to a similar extent as hydrocortisone	Anti-inflammatory	Reuter et al. (2007)
7	30 rats	12 weeks	Administration of diet supplemented with 0.2% or 0.02% rosemary extracts (20% CA)	Rosemary extract decreased cerebral catalase activity, lipid peroxidation and ROS levels, thus protecting the brain, and decreased the activity of catalase and NOS in the heart, protecting this organ	Antioxidant	Posadas et al. (2009)
8	20 rats	12 days	Injection of 3 mg/kg CA daily after surgery	Intraperitoneal injection of carnosic acid could decrease neuronal death	Antioxidant	Azad et al. (2011)
9	24 mice	16 weeks	Administration of low-fat diet, a high-fat-diet or a high-fat-diet with 500 mg rosemary extract/kg body weight per day	Body and epididymal fat weight in animals on the HFD that was supplemented with RE increased 69 and 79% less than those in the HFD group	Against metabolic disorders	Ibarra et al. (2011)
10	13 rats	–	Administration of diet supplemented with 20.5 mg/kg (intravenously) or 64.3 mg/kg (orally)	Bioavailability of carnosic acid, after 360 min, was 40.1%. Traces of carnosic acid found in the rats intestinal content, liver and muscle tissue. Recovery of carnosic acid in the faeces, 24 h after oral administration, was 15.6%	Bioavailability	Doolaege et al. (2011)
11	22 lambs	5 weeks	Administration of barley straw and a concentrate alone (CONTROL group) or enriched with carnosic acid (0.6 g/kg dry matter (DM)), carnosic acid (1.2 g/kg DM) or vitamin E (0.6 g/kg DM)	CA delayed lipid peroxidation in a medium colour-stable muscle (effect lower than with vitamin E), meat texture and protection against cholesterol oxidation (equivalent to vitamin E)	Antioxidant	Morán et al. (2012)
12	30 lambs	56 days	Administration of basal diet or diet enriched with rosemary extracts 1:1 (14–16)% and 2:1 (25–11)% (carnosic acid–carnosol, respectively)	Transfer of CA to lamb meet, favorization of antioxidant status of meat	Bioavailability, antioxidant	Jordán et al. (2014)
13	36 sheeps & 27 lambs	240 days	Administration of basal diet or diet supplemented with 10% or 20% rosemary extract	Rosemary extract induced meat lower levels of lipid oxidation, lower TVC counts and higher colour stability	Antioxidant	Nieto et al. (2010)

response to oxidative stress provoked by drought and high light, isorosmanol (**3**), accumulates in rosemary leaves. Similarly to carnosic acid (**1**) and carnosol (**2**), isorosmanol (**3**) and rosmanol (**5**) were present in chloroplasts but absent from the Golgi apparatus, the endoplasmic reticulum or from the plasma membrane. Their presence in other organelles needs investigation.

9. Conclusions

This review emphasises the importance of and the need for research into edible plants, notably *R. officinalis*, and into their phytochemicals, such as carnosic acid, that exert features valuable for human health and nutrition. There remains a great amount of work urgently needed into the phytochemistry and biochemistry of this and related plants in order to better understand the properties of carnosic acid (**1**) and its role in plants. Beneficial properties have been identified, both for the crude plant extracts and for the constituents, and *in planta* roles can be proposed. Nevertheless, understanding of the mode of action of carnosic acid, both *in vivo* and *in vitro*, is limited, despite the potential that this compound has in a range of applications.

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