Plant Phenylpropanoids as Emerging Anti-Inflammatory Agents

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Abstract: Plant-derived phenylpropanoids (PPPs) compose the largest group of secondary metabolites produced by higher plants, mainly, for the protection against biotic or abiotic stresses such as infections, wounding, UV irradiation, exposure to ozone, pollutants, and herbivores. PPPs are parent molecules for biosynthesis of numerous structurally and functionally diverse plant polyphenols (simple phenolic acids and esters, glycosylated derivatives of primary PPPs, flavonoids, isoflavonoids, stilbenes, coumarins, curcuminoids, lignans, etc.), which play multiple essential roles in plant physiology. During the last few decades, extensive research has been dedicated to natural and biotechnologically produced PPPs for medicinal use as antioxidants, UV screens, anticancer, antiviral, anti-inflammatory, wound healing, and antibacterial agents. In the present review, the metabolic pathways of phenylpropanoid biosynthesis in plants and their re-construction in biotechnologically engineered systems are described. Chemical physical peculiarities of PPPs defining their antioxidant, metal chelating, and UV-protecting effects as a molecular basis for their anti-inflammatory properties are discussed as well. We focused also on the discovery of PPPs-based anti-inflammatory agents since distinct PPPs were found to modulate molecular pathways underlying inflammatory responses in human cells triggered by different pro-inflammatory stimuli *in vitro* and to inhibit inflammation in various tissues *in vivo*. The problem of low bioavailability, fast metabolism, and potential toxicity/sensitization as limiting factors for the development of PPPs-based anti-inflammatory drugs is also highlighted.

Keywords: Aryl hydrocarbon receptor, chemokines, cytokines, reactive oxygen species, UV irradiation, xenobiotics.

INTRODUCTION

Since ancient time plants have been undiminished sources of products traditionally used for medicinal and skin care purposes. In our highly technological era, natural substances of plant origin remain major active principles of numerous drugs and "ceuticals" (nutriceuticals and cosmeceuticals). Thus, approximately 25% of all drugs prescribed today come directly from plants [1]. Many modern drugs derive from parent plant molecules modified by chemical synthesis (semisynthetic drugs). About half of fully synthetic drugs could be considered analogs of natural substances, having a natural product-inspired active moiety [2]. Pharmaceuticals of plant origin are either high (proteins, enzymes, and carbohydrates) or low molecular weight substances, the former belonging mainly to secondary products of plant metabolites [3]. According to a continuously growing mountain of evidence, the beneficial health effects of folk herbal medicine and modern plant-derived medicinal products are attributed to secondary metabolites [4]. Taking into account a vast spectrum of secondary metabolites with medicinal properties, exclusively phenylpropanoids (PPPs) possessing anti-inflammatory properties will be featured in the review.

Great public concern has been raised about the extinguishing plant species sacrificed for the growing drug supply. Since complete chemical synthesis of PPPs is difficult and cost-ineffective, several biotechnological approaches have been developed and applied to produce desired final substances or their precursors. In the present review, we will briefly describe the plant cell/tissue cultures and metabolic engineering approaches as potential biotechnological sources of PPs with anti-inflammatory properties. At the drug discovery stage, several lines of evidence that distinct PPPs may interact with multiple inflammatory pathways hence significantly attenuating inflammatory responses of cells to pro-inflammatory stimuli have been provided [5]. These *in vitro* findings have been confirmed in a number of publications on the PPPs-connected inhibition of inflammation in the *in vivo* animal models [6]. Up-to-now, reliable human studies on anti-inflammatory efficacy of PPPs are few and their results are contradictory [7]. Here, we sought to critically review current literature providing solid basic research and clinical-based evidence that PPPs could be regarded as an emerging class of anti-inflammatory agents for oral and topical administration. We will also discuss serious limitations of PPPs-based anti-inflammatory drug development due to their low bioavailability, fast metabolism, and toxic/allergenic action.

PLANT BIOSYNTHESIS OF PHENYLPROPANOIDS

PPPs are parent molecules for all plant polyphenols, the largest class of secondary metabolites produced *via* shikimic acid pathway [8-9]. The synthesis of PPPs starts from a common initial step – deamination of phenylalanine to cinnamic acid catalyzed by phenylalanine ammonia lyase (PAL, EC 4-3.1-5), a family of enzymes with many isoforms induced by different developmental and environmental stimuli. Several factors are known to affect the expression and activity of the enzyme. Higher plants respond to visible and UV

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light, gamma irradiation, low temperatures, infections [10], wounding [11], germination, toxins like herbicides and pesticides [12] by activation of shikimic acid metabolic pathway, PAL induction, and as a result, increased PPPs biosynthesis. First PPPs family members are cinnamic acid and its close metabolites (hydroxycinnamic acid, 4-coumaric acid, 4-coumaroyl-CoA, caffeic acid, etc.), which form a group of phenolic acids and soluble esters bearing the chemical structure of simple PPPs containing one polyphenolic moiety. They are further catalytically transformed to a large variety of secondary PPPs [8-9,13-15]: (1) glycosylated phenylpropanoids (enzyme: glycosyl transferase), which are structurally characterized by caffeic acid (phenylpropanoid structure) and 4,5 hydroxyphenylethanol (phenylethanoid moiety, hydroxytyrosol) bound to a β-[D]-glucopyranoside through an ester and a glycosidic link, respectively; (2) flavonoids and isoflavonoids (enzyme: chalcon synthase) having flavan nucleus (2 phenylchroman) consisting of 15 carbon atoms, which form three rings (C6-C3-C6), as a basic structure; (3) primary stilbenoids (enzyme: stilbene synthase) and their hydroxylated derivatives; (4) coumarines (enzyme: coumarine synthase); (5) curcuminoids (enzyme: curcumin synthase); and (6) phenolic polymers such as tannins (synonymous to proanthocyanidins), suberins, lignins, and lignans (enzymes: plant peroxidases) formed in the reaction of oxidative coupling of polyphenol monomers [8-9,14-16]. In the Fig. (**1**) we design a genealogical tree and present several chemical structured of plant polyphenols produced through phenylpropanoid biosynthetic pathway.

PHYSICAL CHEMICAL PROPERTIES OF PHENYLPROPANOIDS AS A BASIS FOR THEIR PHYSIOLOGICAL ROLES IN PLANTS AND MAMMALS

The superfamily of PPPs (more than 4,000 substances have been described so far [17]) is composed of numerous, structurally and functionally diverse and, nevertheless, biologically similar active substances. Their similarity is determined by the unique parent molecule and by the presence of phenoxyl groups, which largely determine their peculiar chemical and photo-chemical reactivity. Numerosity of PPPs hints on the evolutionary diversification of their functions in plant physiology. In general, PPPs and distinct other plant polyphenols appear to protect plants against physical, chemical, and biological damage providing an appropriate defense and adaptation to hostile environment, pathogens, herbivores, and competitor plant species. Moreover, they are essential for reproductive advantages as attractants of pollinators and seed dispersers as well as for competitive advantages poisoning rival plants and predators. There seem that PPPs play multiple roles in the maintenance of plant homeo-

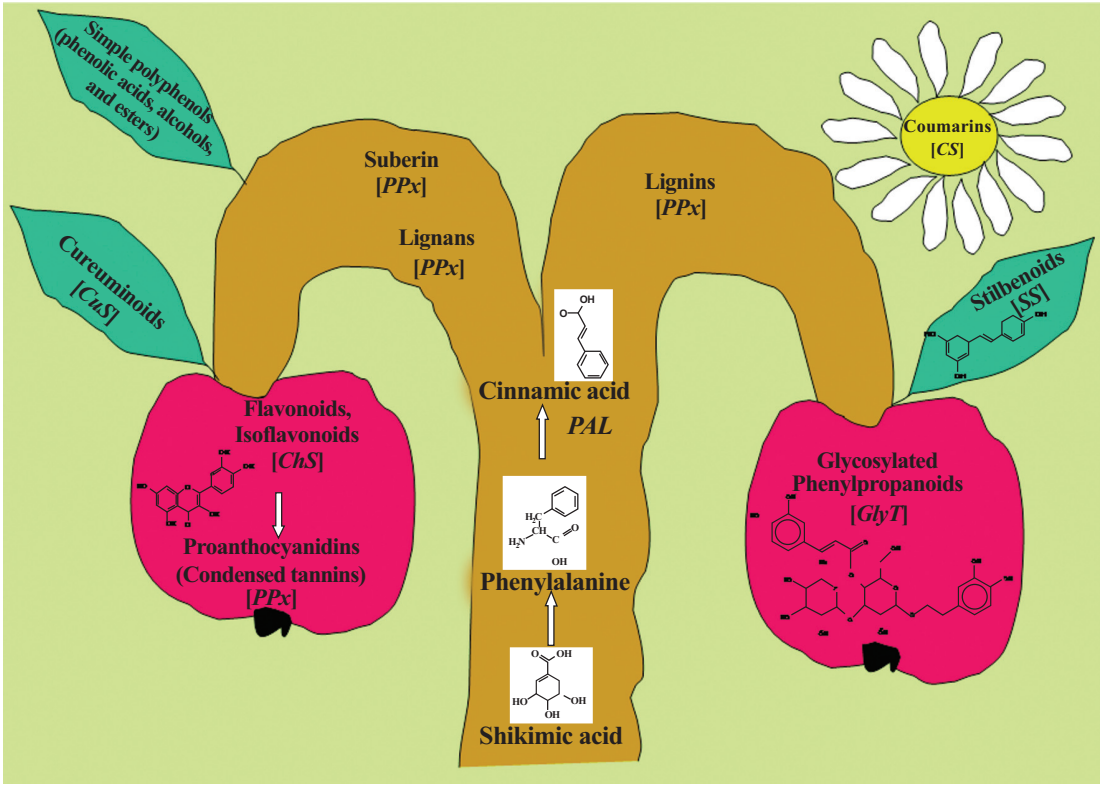


Fig. (1). Phenylpropanoid pathway of biosynthesis in higher plants.

The unique precursor – shikimic acid is transformed into amino acid phenylalanine, which in its turn is metabolically converted into first phenylpropanoid structure of cinnamic acid by phenylalanine lyase (*PAL*), an inducible enzymes reacting to biotic and abiotic stresses. All groups of plant polyphenols derive from a parent simple phenylpropanoid molecule in a variety of enzymatic reactions: glycosylated phenylpropanoids are formed in glycosyl transferase [*GlyT*] catalyzed reactions, stilbenoids – in stilbenoid synthase [*SS*] catalyzed reactions, coumarines – coumarine synthase catalyzed reactions [*CS*], flavonoids and isoflavonoids – chalcone synthase [*ChS*] catalyzed reactions, curcuminoids – curcumine synthase [*CuS*] catalyzed reactions, and polyphenolic polymers like proanthocyanidins, lignins, lignans, and suberin are formed either in plant peroxidase [*PPx*] catalyzed reactions or non-enzymatically.

stasis likewise chemical defensive, immune, nervous, and endocrine systems do together in mammals. With the progress of molecular biology, multiple other functions of PPPs and their metabolic "off-springs" in the intracellular and cell-to-cell signaling as well as in the plant growth and differentiation control have been recognized and summarized in comprehensive reviews and books [18-20]. Being metabolic derivatives of amino acid phenylalanine, PPPs share some structural and functional similarities with numerous biologically active mammal molecules synthesized from phenylalanine as a precursor, such as catecholamines, thyroid and estrogen hormones, melanin moieties, etc. [4]. Hence PPPs could easily interfere with metabolic processes and molecular pathways in mammal/human cells/tissues bringing either desired health or adverse toxic effects.

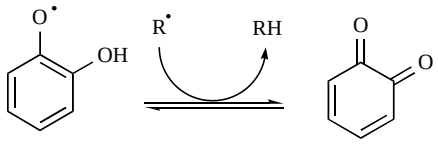
Redox Properties of PPPs

Distinct peculiar physical chemical properties underlie important physiological functions of PPPs in the host plants and biological effects towards mammal cells and tissues. Practically all PPPs under physiological conditions possess reduction/oxidation (redox) properties, thus exerting direct antioxidant or pro-oxidant effects. Their low redox potentials usually range between $E°$ 0.25 – 0.75V [21-23] hence they easily donate one electron to compounds with higher redox potential, for example free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl radical, ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals. The outcome of these reactions is free radical scavenging:

$$Ph\text{-}OH + R^{\bullet} \rightarrow Ph\text{-}O^{\bullet} + RH \quad (1),$$

where Ph-OH and Ph-$O^{\bullet}$ are the phenolic molecule and the phenoxyl radical, respectively; and $R^{\bullet}$ is a free radical.

The phenoxyl radical may react with a second free radical acquiring a stable ***o***-quinone structure:



(2)

The high reactivity of PPPs and phenoxyl radicals toward different chain initiating radicals or peroxyl radicals (equations 1 and 2) underlies the chain-breaking mechanism of their antioxidant activity. A great majority of anti-inflammatory and other beneficial health effects of PPPs has been traditionally attributed to their direct antioxidant properties (For comprehensive reviews see [24-29]).

Alternatively, in the presence of molecular oxygen, PPPs with low redox potentials may be easily oxidized through a free radical-driven chain autoxidation process, described by the equations 4-8 [30-31].

$$RH_3^- + O_2 \rightarrow RH_3^{\bullet} + O_2^{\bullet-} + H^+ \quad (4)$$

$$RH_3^- + O_2^{\bullet-} + 2H^+ \rightarrow RH_3^{\bullet} + H_2O_2 \quad (5)$$

$$RH_3^{\bullet} + O_2 \rightarrow RH_2 + O_2^{\bullet-} + H^+ \quad (6)$$

$$RH_2 + O_2^{\bullet-} + H \rightarrow RH^{\bullet} + H_2O_2 \quad (7)$$

$$RH^{\bullet} + O_2 + H \rightarrow R + O_2^{\bullet-} + H^+ \quad (8)$$

Transition metals (Me^{n+}) could be involved in the initiation of polyphenol autoxidation as catalysts of reaction 4 (equations 9 and 10):

$$RH_3^- + Me^{n+} \rightarrow RH_3^{\bullet} + Me^{(n-1)+} \quad (9)$$

$$Me^{(n-1)+} + O_2 \rightarrow Me^{n+} + O_2^{\bullet-} \quad (10)$$

Numerous polyphenols were found to be strong chelators of metal ions, such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} [32-35]. The redox reactions of metal-polyphenol complexes depend on the nature of ligand, strength of the metal-ligand bond, and the redox potential of the complex [13, 64, 65].

$$Ph[Me^{(n+1)+}] + O_2^{\bullet-} \rightarrow Ph[Me^{n+}] + O_2 \quad (11)$$

$$Ph[Me^{n+}] + O_2^{\bullet-} + 2H^+ \rightarrow Ph[Me^{(n+1)+}] + H_2O_2 \quad (12)$$

$$H_2O_2 + Ph[Me^{n+}] \rightarrow {}^{\bullet}OH + {}^{-}OH + Ph\,[Me^{(n+1)+}] \rightarrow 2^{-}OH + Ph_{ox}\,[Me^{(n+1)+}] \quad (13)$$

$$Ph[Me^{n+}] + O_2 \rightarrow Ph\,[Me^{(n+1)+}] + O_2^{\bullet-} \quad (14)$$

$$H_2O_2 + Ph[Me^{n+}] \rightarrow {}^{\bullet}OH + {}^{-}OH + Ph\,[Me^{(n+1)+}] \quad (15)$$

From the above reactions it is clear that the formation of Me-PPP complexes may cause crucial changes in both the transition metal and PPPs redox properties: (1) Fe^{+2} and Cu^{+2} bound into complexes are not anymore able to catalyze hydroxyl radical formation *via* the Fenton reaction [21, 36-38]; (2) complexes react with superoxide radicals in a superoxide dismutase-mimicking manner (equations 11 and 12) [39] or with H_2O_2 in a peroxidase-like fashion (equation 13) [40]; and (3) complexes react with molecular oxygen or hydrogen peroxide producing superoxide or hydroxyl radicals, respectively (equations 14 and 15) [21,41]. If the first two reactions demonstrate enhanced antioxidant/free radical scavenging capacity of complexes as compared to their single components, the last three reactions show alternative free-radical generating and hence, potential pro-oxidant effects. For example, complexes of rutin, dihydroquercetin, and green tea epicatechins with Fe^{2+}, Fe^{3+}, or Cu^{2+} possess superoxide scavenging (reactions 11 and 12) and catalase-like hydrogen peroxide-decomposing properties (reaction 13) more pronounced than the parent PPPs molecules [33, 34, 39-41]. On the other hand, several flavonoids, curcumin, resveratrol, and epigallocatechin gallate can mobilize copper ions from chromatin and form redox active complexes (reactions 14 and 15) in a close vicinity to DNA, thus causing DNA cleavage [42, 43].

Primary semiquinones or phenoxyl radicals could be also produced enzymatically in the reactions of polyphenols with peroxidase/H_2O_2 or cytochrome P-450 [44-46]. Once primary radicals are formed, they react in a chain-like manner converting PPP to a quinone structure during the propagation stage (equations 6-8, PPPs-driven catalytic redox cycling process). Termination of the chain autoxidation of polyphenols takes place either due to the disproportionation of semiquinones, phenoxyl and superoxides radicals, or to the interaction of radicals with redox-active transition metals [21].

Short-lived reactive by-products of PPPs autoxidation, such as semiquinones and ROS, besides being initiators and intermediates of free radical-driven PPP redox cycling, may

interact with biomolecules, initiating lipid peroxidation, protein and DNA oxidation, formation of DNA adducts, and endogenous antioxidant depletion [46, 47] followed by membrane damages, changes in enzymes and receptors, mutations, and extracellular matrix destruction. This corresponds to a traditional view on the oxidative stress-associated cell damage, for example that driven by exaggerated acute or long-lasting chronic inflammation [48]. On the other hand, low levels of such by-products may initiate adaptive cellular response by activating redox signal transduction pathways [49] thus facilitating induction of endogenous antioxidant and detoxifying systems [50], cessation of inflammation [51], and repair of cell/tissue damage [52]. The latest data and based on them emerging views/hypothesis indicate that direct "chemical" antioxidant properties of PPPs cannot fully explain their anti-inflammatory and other cell/tissue protective effects. Rather, these effects seem to depend on indirect induction of endogenous antioxidant defense by PPPs [53, 54].

In conclusion, PPPs, depending on their dose, chemical structure, one electron redox potential, and the redox properties of the environment may exert either free radical scavenging / antioxidant or free radical generating / pro-oxidant action. Depending on the intensity, duration, and location of PPPs-associated redox processes, direct oxidative damage or positive adaptive reactions may occur. Alternative biological outcomes (mainly connected with inflammation) of PPPs redox reactions are schematically represented in Fig. (**2**).

Interaction of PPPs with UV Irradiation

It has been shown that many plants respond to enhanced UV radiation by increasing the concentrations of UV absorbing compounds in the epidermal cells. Many PPPs, first of all, hydroxycinnamic acid and cinnamoyl esters, then, flavones, flavonols, and anthocyanins, are synthesized by higher plants to provide UV-A and UV-B screening for vital but not essential for photo-synthesis compartments of plant cells because they absorb efficiently UV light in the range of 304-350 nm and 352-385 nm, respectively [4]. The absorption spectra and extinction coefficients of PPPs are influenced by their structure, electron-accepting and electron-donating substitutents in the benzene ring(s), intra- and intermolecular hydrogen-bonding, and by steric effects [55].

The damaging effects of UV light towards plant and mammalian cells occur either directly by attacking biologically important molecules such as DNA (Type I reaction) or *via* the generation of reactive oxygen species (ROS) such as singlet oxygen and superoxide anion-radical (Type II reaction) [56]. In general, UV light is primarily absorbed by endogenous chromophores (trans-urocanic acid, melanins, por-

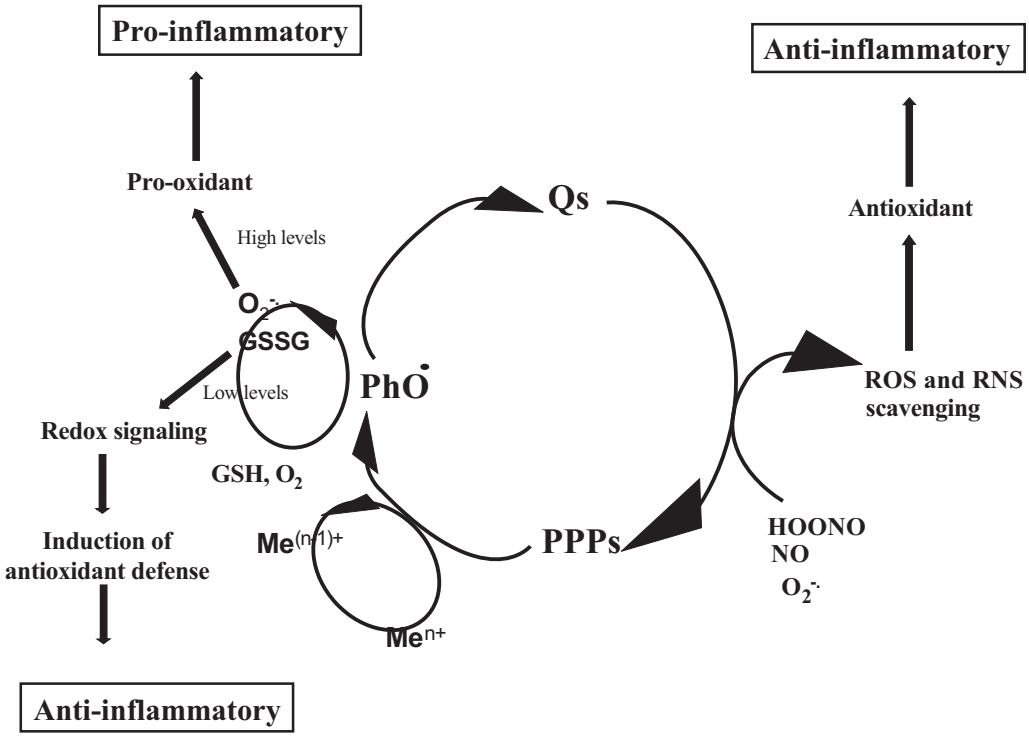


Fig. (2). Redox-dependent anti- and pro-inflammatory effects of plant phenylpropanoids Phenylpropanoids (PPs) react with reactive oxygen (ROS, $O_2^{\cdot-}$) and reactive nitrogen (RNS, NO and peroxynitrite HOONO) providing their effective scavenging followed by chain-breaking inhibition of oxidative reactions in lipids (antioxidant action). Therefore, the levels of non-protein mediators of inflammation, such as ROS, RNS, lipid peroxides, and lipid-derived aldehydes diminish, thus anti-oxidant and anti-inflammatory effects take place.

PPPs, in the presence of transition metals or enzymatically, may be converted into highly reactive phenoxyl radicals ($PhO^{\cdot}$), which interact with molecular oxygen and reduced form of glutathione (GSH). As a result, phenoxyl radical acquire stable quinone structure (Qs) and superoxide anion-radical or oxidized glutathione (GSSG) are formed. While low levels of superoxide facilitate redox signaling leading to the induction of endogenous antioxidant enzymes followed by inhibition of inflammation, high levels of superoxide cause pro-oxidant damage to biomolecules and inflammatory response to the damage.

phyrins, flavins, quinones, chlorophil, plant pigments, tryptophan, hydropiridines, bilirubin, and glycation end-products) [57]. Of note, majority of PPPs belong to plant pigments hence effective chromophores for UV light. After absorption of the light energy, the chromophores become activated (excitation state). The activated chromophores may dissipate excessive energy in exothermal process by emitting infrared radiation, thus playing an essential role of chemical UV filters. Alternatively, they directly react with a target molecule, thus transforming it in a corresponding free radical (Type I photo-sensitization) [58]. In the presence of oxygen, its reaction with activated chromophore takes place and superoxide radicals or singlet oxygen are formed. These ROS may induce oxidative modifications of lipids, proteins, and DNA (Type II photo-sensitization). Collectively, PPPs participate in photo-chemical reactions and, depending on their chemical structure and concentration, may diminish UV damage (photo-protective PPPs) or aggravate it (photo-toxic PPPs).

BIOLOGICAL ACTIVITIES OF PHENYLPROPANOIDS RELEVANT TO INFLAMMATORY RESPONSES IN HUMANS

Numerous reports indicate that PPPs and their polyphenolic derivatives exert anti-inflammatory activity, as demonstrated in animal models or in cultured cells [59-67]. Indeed, 12 out of 40 anti-inflammatory drugs approved between 1983 and 1994 worldwide were derived from or based on natural PPPs [62]. The best example is aspirin (acetyl salicylic acid), the most frequently used nonsteroidal anti-inflammatory drug. Mechanistic studies have repeatedly shown that PPPs with free radical scavenging, antioxidant, and transition metal chelating activity may directly diminish levels of non-protein inflammatory mediators such as free radicals, hydrogen peroxide, lipid peroxides, and aldehydes, final products of lipid peroxidation, such as malonyl dialdehyde, 4-hydroxy-2-nonenal, acrolein, and others [21,36,60].

As documented by a vast literature, PPPs inhibit major pro-inflammatory enzymes such as inducible nitric oxide synthase (iNOS), NADPH oxidase, eicosanoid-generating enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), and also phospholipase A_2, which liberates arachidonic acid from membrane-bound phospholipids [60,62,68-69]. Hence inhibition of this last enzyme prevents generation of arachidonic acid-derived mediators of inflammation. Furthermore, interaction of PPPs with distinct receptors such as peroxisome proliferator activated receptors (PPARs) and estrogen receptors (ERs) results in the inhibition of inflammatory responses [62,70-71] through suppression of inflammatory gene transcription [71-73]. Distinct PPPs, such as resveratrol, capsaicin, curcumin, epigallocatechin galate, genistein, and others [62] are inducers of non-steroidal anti-inflammatory drug activated gene-1 likewise non-steroidal anti-inflammatory drugs.

Numerous experimental data confirm that PPPs exert their anti-inflammatory action by modulating cellular inflammatory response regulated mainly by nuclear factor kappaB factor (NFκB) [59,73]. As a consequence, the NFκB-dependent expression of pro-inflammatory proteins-cytokines such as IL-1, IL-6, GM-CSF and TNF-α is dramatically impaired or totally blocked [74]. Substantial experimental evidence points out that PPPs, for example, apigenin and curcumin, act as potent inhibitors of activator protein (AP-1), a transcription factor controlling stress responses in a variety of human cells [59,75]. Moreover, PPPs-induced abrogation of both factors NFκB and AP-1 provides the best condition to oppose expression of pro-inflammatory mediators and may have a great impact on their anti-inflammatory action [76].

Many regulatory elements presumably implicated in the inflammatory cell responses, such as the antioxidant response element (ARE) [77], protein kinase B (Akt), and extracellular signal-regulated protein kinase1/2 (ERK1/2) [78,79] are involved in signal transduction from PPPs to PPPs -responsive genes. For comprehensive reviews on the subject see [80,81].

Major mechanisms implicated in the PPPs-mediated chemoprevention from inadequate inflammatory response or inhibition of intensity or/and duration of on-going inflammation (anti-inflammatory action) are schematically shown in Fig. (**3**).

NATURAL AND BIOTECHNOLOGICALLY-PRODUCED PHENYLPROPANOIDS-INHIBITORS OF SKIN INFLAMMATION

Traditionally, PPPs are extracted and purified from harvested plant parts. However, the industrial development of medicinal and "ceutical" products based on the extraction and purification of PPPs from plant material is limited due to several reasons: (i) low abundance in nature and seasonal variations in plant harvesting, (ii) concern about shrinking biodiversity, (iii) contamination of plant parts with herbicides, insecticides, growth hormones, and environmental pollutants, (iv) due to variable conditions for plant growth, and (v) difficulties of PPPs extraction and purification from the parts of grown plant, thus poor standardization of the final product.

Plant cell/tissue cultures seem to be an ideal "chemical factory" for the production of desired substances because they overcome all the above limitations providing controlled, uncontaminated, and all year round biosynthesis of complex molecules. Since PPPs molecules can be released from plant cells into culture medium, their extraction and purification are greatly simplified. Major shortcomings of plant cell/tissue-based production systems remain lower yields as compared with a whole plant, biochemical and genetic instability of cultured plant cells, and scale-up problems. Up to now, there are only a few examples of successful PPPs production in stabilized callus/tissue cultures established from medicinal herbs with ethnobotanically recognized anti-inflammatory properties, such as verbascoside, teupolioside, chlorogenic acid, and esters of caffeic acid are produced in industrial quantities. Another promising perspective is genetic engineering of PPPs biosynthesis in microbes and yeasts, classical systems for industrial high-yield productive process [82]. However, the extreme complexity of PPPs biosynthesis and the lack of knowledge on the entire process are two major obstacles for successful plant gene expression in microbes/yeasts.

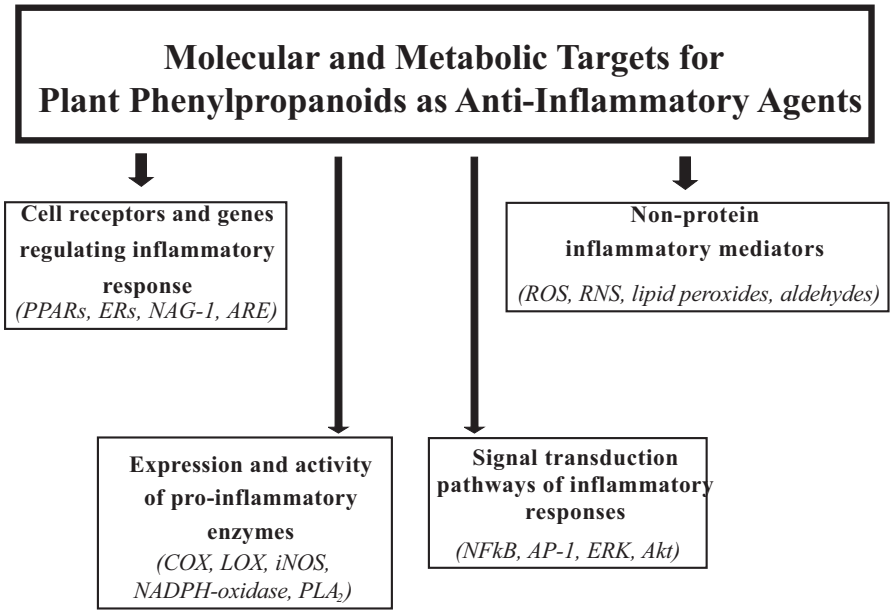


Fig. (3). Multiple molecular and metabolic targets for plant phenylpropanoids as anti-inflammatory agents.

Extensive search for new effective agents to prevent and combat acute and chronic skin inflammation indicated a number of natural and biotechnologically produced PPPs. Particular interest has been paid to the PPPs-inhibitors of UV-induced chronic inflammation that could prevent UV-related skin tumors and premature skin aging.

Effects on Chemokine and Cytokine Expression

Physical, chemical, or immune-specific insults rapidly evoke an epidermal response characterized by the increased expression of a system of pro-inflammatory mediators, including chemotactic factors, which initiate the orientated migration of distinct leukocyte subpopulations. In turn, activated monocytes, dendritic cells and in particular stimulated T cells release potent cytokines that act on cells in the local environment to boost the protective inflammatory response [83]. In particular, tumor necrosis factor (TNF)-α and interferon (IFN)-γ induce the expression of numerous chemokines, including monocyte chemoattractant protein (MCP)-1, IFN-γ-induced protein of 10 kilodalton (IP-10), and interleukin (IL)-8, and growth factors such as granulocyte/macrophage-colony stimulating factor (GM-CSF) in keratinocytes. Epidermal MCP-1 is involved in the early response to injury or irritants, and in T cell-mediated skin disorders, and controls the recruitment of monocyte-macrophages, dendritic cells and T cells. However, T cells appear massively attracted into the skin by keratinocyte release of IP-10. This T cell-selective chemokine is deeply involved in T cell-mediated diseases such as allergic contact dermatitis and psoriasis, but is not relevantly expressed during skin response to irritants [76]. Finally, IL-8 is the best characterized of a group of chemoattractants active in neutrophil recruitment as well as in epithelial and endothelial cell proliferation, whereas GM-CSF is recognized as a major immune regulator governing survival of granulocyte and macrophage lineage populations at all stages of maturation [84]. At variance with the chemokines MCP-1 and IP-10, whose *de novo* expression is strictly dependent on the activation of the transcription factor NFκB, IL-8 and GM-CSF can be induced by the only activation of the transcription factor activator protein (AP)-1, although their maximal expression requires the cooperation of AP-1 and NFκB [76-84]. AP-1 activation depends on the mitogen-activated protein kinases (MAPKs), which include extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 α and β MAPKs and the c-Jun NH2-terminal kinase/stress-activated protein kinase 1 and 2 (JNK1/2) [85]. In human keratinocytes, ERK1/2 is expressed at very high levels, and is constantly kept in an activated state by epidermal growth factor receptor (EGFR) signaling [86], whereas p38 MAPKs and the JNKs are transiently stimulated on exposure to pro-inflammatory cytokines [85]. Indeed, through its persistent induction of ERK1/2, EGFR governs the homeostatic maintenance and repair of epithelial tissues, including up-regulation of IL-8 and GM-CSF, and down-regulation of the chemokines MCP-1 and IP-10 [86].

PPPs may exert protective anti-inflammatory activity on the skin, as demonstrated in animal models or its cell populations in culture [56]. In a recent report, we demonstrated that the glycosylated PPPs verbascoside and teupolioside could efficiently and dose-dependently oppose the release of a cluster of pro-inflammatory mediators including the cytokine GM-CSF and the chemokines IL-8, IP-10, and MCP-1, triggered by TNF-α alone or its combination with IFN-γ [4]. In a preliminary comparison with equimolar concentrations of the flavonoid rutin and its aglycone quercetin, we confirmed that verbascoside was the most potent inhibitor of IL-8 and IP-10 expression, and we could observe that this compound was the most potent inhibitor of both NFκB and AP-1 transactivation [87]. Indeed, a vast literature suggests that a number of dietary polyphenols, including the bioflavonoids rutin and quercetin, and the stilbenoid resveratrol may remarkably inhibit the intracellular signalling pathways leading to pro-inflammatory cell activation. Importantly, although these activating mechanisms are present in all resident and im-

mune cells, there are relevant, cell type-specific differences in the anti-inflammatory effects of PPPs [59,88]. In the next paragraphs, we will comment on our experimental evidence of the impact of these xenobiotics on the major signal transduction events recognized as centrally involved in the pro-inflammatory cell response in human keratinocytes.

Effects on NFκB

A plethora of *in vitro* and *ex vivo* experimental data confirm that PPPs exert their anti-inflammatory action by modulating crucial cellular signalling processes, in particular those leading to NFκB activation [59,88]. NFκB is inactive when bound to IkappaB in the cytosol. Following a variety of stimuli in which reactive oxygen species operate as pathway intermediates [89], the phosphorylation of IκB by IκB kinases (IKKs) leads to proteasome-dependent degradation of IκB, allowing the transactivating NFκB subunits free to enter the nucleus and induce the expression of genes involved in cell cycle progression and active leukocyte recruitment [88,90]. In the whole animal skin and in its distinct cell populations, polyphenols inhibit NFκB activation by blocking IKK activity, hence inhibiting IκB phosphorylation and degradation. As a consequence, the NFκB-dependent expression of primary pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α is dramatically impaired [74]. Treated with a variety of polyphenols, which include silymarin [91], resveratrol [92], (-)-epigallocatechin-3-gallate [93], or the extract of pomegranate fruit [94], also human keratinocytes are protected from UVB-induced up-regulation of NFκB activity.

We have recently observed that a cluster of polyphenols including the glycosylated PPP verbascoside, resveratrol and its glycosylated derivative polydatin, quercetin and its glycosylated derivative rutin, were all effective inhibitors of NFκB activation due to stimulation TNF-α stimulation, a molecular event that preceded and correlated with the dose-dependent down-regulation of the NFκB-restricted chemokines MCP-1 and IP-10, both in normal human keratinocytes and in the spontaneously immortalized keratinocyte cell line HaCaT [95]. Similarly, these PPPs impaired the pro-inflammatory response of normal human keratinocytes to the mixture of TNF-α and IFN-γ, with early suppression of NFκB activation and consequent dose-dependent down-regulation of IP-10 release [96]. This body of evidence indicates that, beside NFκB perturbation, these substances may affect the inflammatory response of human keratinocytes in a complex, non-univocal fashion, depending on their chemical structure but independent on their anti-oxidant properties. Hence, a number of NFκB-independent pathways reasonably contribute to the final effect of polyphenols on keratinocyte pro-inflammatory response.

Effects on Epidermal Growth Factor Receptor-Extracellular Response Kinase (EGFR-ERK) Pathway

If PPPs can be considered as effective NFκB inhibitors [88,90], they cannot be invariably identified as AP-1 inhibitors. Indeed, red-ox perturbation of cell environment may have opposite effects on the activation of NFκB and AP-1 [97]. AP-1 is a heterodimeric transcription factor mainly linked to growth/differentiation homeostasis, and its dysregulation is implicated in UVB-mediated cell transformation and tumor promotion [98]. Importantly, AP-1 activity undergoes a complex, cell type-specific regulation. The DNA binding activity of its subunits jun and fos, is regulated by posttranscriptional mechanisms involving reduction-oxidation, with oxidation having in general an inhibitory effect and reduction a stimulatory effect [99]. In addition, both the synthesis and the functional state of AP-1 subunits are under the control of distinct classes of MAPKs, in their turn also red-ox sensitive. In particular, reactive oxygen species tend to potentiate MAPK cascade not only by direct activation, but also by inhibition of de-activating phosphatases, globally leading to upregulation of AP-1 activity [85,100]. In particular, in human keratinocyes, a sustained, EGFR-driven activation of ERK1/2 guarantees high steady-state levels of AP-1 activity and, in the presence of pro-inflammatory stimuli, also prevents from abnormal expression of secondary mediators including MCP-1 and IP-10 [76]. In contrast with the experimental evidence collected from a variety of cell types, in which polyphenolic antioxidants act as potent ERK inhibitors [59], human keratinocytes respond to (-)-epigallocatechin-3-gallate with a ERK activation. By contrast, the flavonoid apigenin and curcumin potently inhibit this signalling pathway [75].

We found that, either used alone or in association with TNF-α, verbascoside, the stilbenoids resveratrol and polydatin, and the flavonoids rutin and quercetin were all potent inhibitors of ERK phosphorylation in normal human keratinocytes, with verbascoside and quercetin showing the strongest activity, since they totally abrogate phosphorylated ERK signal [95]. By contrast, in the same experimental conditions, the spontaneously transformed cell line HaCaT was less sensitive to these PPPs in terms of impairment of ERK activation. Specifically, in this cell line, only quercetin maintained its inhibitory effects both in unstimulated and TNF-α-stimulated conditions, whereas verbascoside effectively opposed ERK activation only in unstimulated conditions. More relevant, we observed that, either used alone or in association with UVA irradiation or with lipopolysaccharide, these PPPs prominently up-regulated the transcript levels of a number of inflammatory mediators at early time-points, including IL-6, IL-8 and TNF-α in HaCaT cells but not in normal human keratinocytes [95]. This dramatic discrepancy, and the evidence that HaCaT cells respond to TNF-α with a much stronger NFκB activation, highlight that HaCaT cells should not be considered a valid model to routinely investigate the response of primary human keratinocytes to xenobiotics.

More recently, we further investigated the effects of PPPs on EGFR phosphorylation and their possible relation with the final ERK levels in normal human keratinocytes. Indeed, a number of PPPs, including quercetin, have been reported to effectively display anti-EGFR, and in general anti-tyrosine kinase receptor activities [101]. We reproducibly found that only verbascoside and quercetin, the most active ERK inhibitors, were also effective in the inhibition of EGFR phosphorylation, either used alone or in combination with TNF-α and IFN-γ (own unpublished data). This observation suggests that the profound inhibition of ERK activity due to verbascoside and quercetin may depend on the combination of both a direct, ERK-targeted, and an indirect, EGFR-

dependent inhibition of ERK phosphorylation in normal human keratinocytes. Importantly, this molecule-specific profile of activity is totally uncorrelated to the anti-oxidant profile of these substances.

Effects on Aryl Hydrocarbon Receptor (AhR)

Beyond their well-investigated activity on NFκB and MAPKs, PPPs can affect gene expression through a variety of other signaling pathways, whose impact on the expression of inflammatory mediators has been investigated only marginally so far. In particular, being aromatic hydrocarbons, these substances can bind the AhR transcription factor and contribute to the transcription of numerous detoxification genes coding for phase I and II metabolizing enzymes, particularly the cytochrome P450 CYP1 subfamily, Nrf2, and glutathione S-transferase (GST) [60,102,103]. Importantly, current knowledge indicates an ample array of biological functions for AhR, far beyond its classical role in detoxification. AhR is involved in cell differentiation and cycling, wound healing, immune responses, ageing, and cancer promotion, and this happens also through the control of the expression of a number of pro-inflammatory factors, such as cytokines and growth factors [104-106]. In addition, it is thought that AhR machinery is a "master switch" of cell responses to various stresses. The AhR is a ligand-activated transcriptional factor shuttling from cytoplasm to nucleus upon activation [104]. After translocation to nucleus, AhR binds to its heteromerization partner AhR nuclear traslocator (Arnt) and this complex finally binds to xenobiotic responsive elements (XRE) in the promoter region of the target genes. Apart from CYP, phase II enzymes, and Nrf2, growth factors, cytokines and their receptors are major downstream targets for AhR-connected signaling pathways [105,107]. Importantly, AhR is interconnected with multiple signal transduction pathways, since (a) it has several functional connections with EGFR pathway [108], (b) a two-way interconnection with mitogen activated protein kinases MAPKs, especially with ERK has been documented [109,110], and (c) close interplay between AhR and NFκB exists [109]. As a consequence, AhR could reasonably contribute to control the expression of major players of inflammatory responses also in keratinocytes. Several PPPs, such as the isoflavone genistein and the flavonoid Qr have been identified as AhR ligands, whereas others antagonized with this receptor [111] or, depending on concentration and PPPs structure, could be both agonists and antagonists [112].

We recently observed that, although verbascoside, resveratrol and polydatin, rutin and quercetin significantly reduced the nuclear translocation, and hence the total amount of nuclear AhR in unstimulated conditions, nonetheless resveratrol, polydatin and rutin enhanced AhR nuclear levels when administered in combination with UV stimulation or with the tryptophan metabolite, an endogenous AhR ligand formed upon exposure to UVB [108]. By contrast, the strong ERK inhibitors verbascoside and quercetin acted by suppressing AhR nuclear translocation due to UV or its ligand (own unpublished data). Again, these preliminary data indicate that PPPs act distinctly on the AhR system. These results suggest that PPPs might operate their articulate control on cytokine/chemokine expression also *via* AhR-mediated molecular processes. Fig. (**4**) demonstrates a complex network of inflammatory pathways in human keratinocytes targeted by PPPs.

Anti-inflammatory action of PPPs have been largely attributed to their classical chain-breaking antioxidant or free radical scavenging activities. However, in the last 5-10 years, evidence from numerous *in vitro* skin cell studies suggests that they can influence cellular functions by multiple other mechanisms, such as direct interaction with several receptors, modulation of intracellular signal transduction and transcription of a number of genes, post-translational modulation of enzymatic activities as well as epigenetic regulation of gene expression [113-115]. During the last decade, the idea of PPPs-connected transcriptional up-regulation of antioxidant and detoxifying enzymes through nuclear factor erythroid-derived 2 (NFE2)-related factor (Nrf2) pathway as an evidence for their indirect "antioxidant" activity has gained grounds [116, 117].

Effects on UV-Induced Inflammation

Experiments in mice suggest that a variety of PPPs extracted from green tea, grape seed, soy beans, or *Curcuma longa* root, when taken orally or applied topically, do exert chemoprevention of skin inflammation (erythema and sunburned lesions) followed by a significant decrease in non-malignant and malignant skin tumors derived from exposure to carcinogenic doses of UV rays [118,119]. In a study on a small number of human subjects, the soybean isoflavone genistein effectively inhibited UVB-induced erythema when applied onto the skin before UVB irradiation [120]. PPPs-containing phytochemicals directly exert their chemoprevention of properties by diminishing the levels of pro-inflammatory mediators such as free radicals, inorganic and organic peroxides, and aldehydes generated by UV irradiation [14-15]. Oxidation end-products of sebum and keratinocyte cell membrane lipid moieties represent key biochemical mediators of the inflammatory reaction induced by UV irradiation [121-123], among which 4-hydroxynonenal (4-HNE) is the most thoroughly characterized. This stable biomarker of polyunsaturated lipid oxidation is produced in vitiligo keratinocyte membranes, where its abnormal levels may cause EGFR dysfunctions and consequent perturbation of cytokine patterns, whilst abnormal plasmatic levels of 4-HNE are found in other chronic inflammatory autoimmune skin diseases with elevated lipid peroxidation features, such as *lupus erythematosus* [124]. Green tea polyphenols, theaflavins, and other PPs acting as sacrificial nucleophiles have been proven to trap 4-HNE efficiently, the reason why tea and red wine polyphenols protect various types of cells *in vitro*, against HNE-induced intracellular oxidative stress and cytotoxicity [125]. In addition, a significantly inhibition of HNE-induced mitochondrial ROS production is afforded by quercetin, EGCG, theaflavins and their gallate esters [126]. The most documented applications for skin damage protection under photo-irradiation anti-proliferative therapy concern standardized green tea extracts, due to their potent anti-inflammatory and photoprotective properties [93]. Green tea epicatechin derivatives, possessing free radical scavenging, antioxidant, anti-inflammatory, as well as direct anti-carcinogenic properties, have shown to be protective against UVB- or PUVA-induced photo-toxicity, with inflammation control through the suppression of proteasome function, NF-

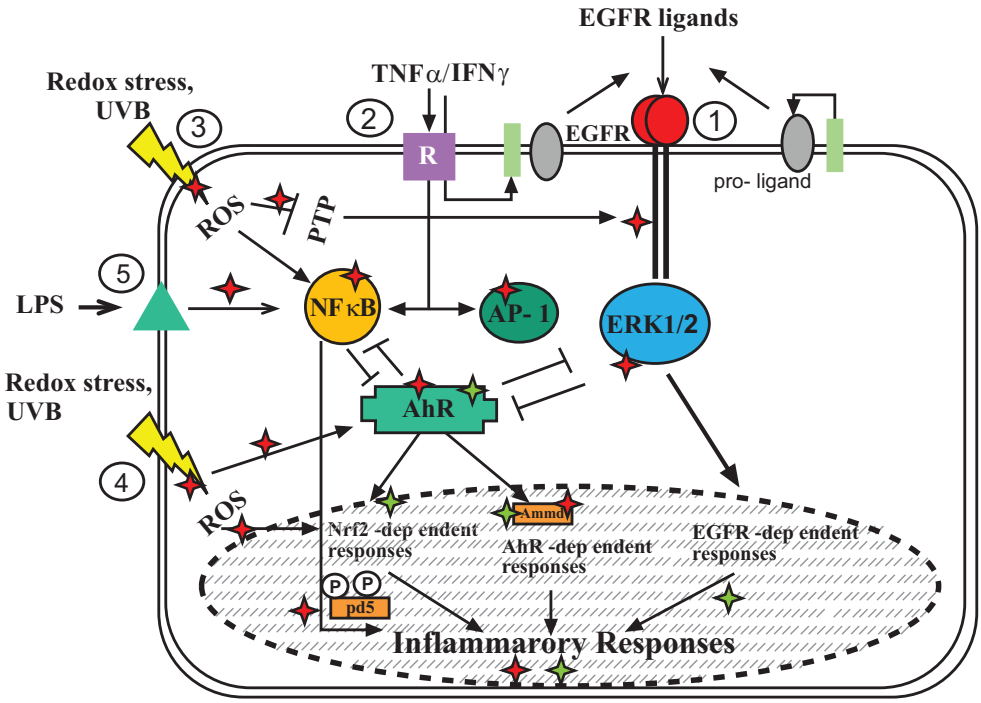


Fig. (4). Inflammatory pathways modulated by plant phenylpropanoids in human keratinocytes. Pathway 1 (in circle) connected with direct activation of epidermal growth factor receptor (EGFR) by ligands is inhibited by PPPs (red asterisks), which may result in the enhancement of EGFR-regulated inflammatory responses (green asterisks).

Pathway 2 (in circle) connected with membrane receptors (R) for pro-inflammatory cytokines TNFα and IFNγ converges with Pathway 1 at the levels of EGFR pro-ligand activation. Stimulation of R by the combination of TNFα/IFNγ activates NFκB and AP-1 transcriptional factors, which target genes coding pro-inflammatory cytokines and enzymes. PPPs may inhibit either NFκB or AP-1 or both transcription factors (red asterisks). NFκB and aryl hydrocarbon receptor (AhR) exhibit suppressive action towards each other, hence PPs reacting directly with AhR may dysregulate NFκB pro-inflammatory pathway. Pathway 3 (in circle) depends on reactive oxygen species (ROS) formed intracellularly under UV-irradiation or exogenous oxidative stress conditions (X-rays, toxins, heavy metals, etc.). ROS-mediated inhibition of protein tyrosine phosphatase (PTP) maintains EGFR in an activated state, thus interfering with Pathway 1. ROS induce also redox-sensitive transcription factors, NFκB first of all. PPPs may interfere with this pathway absorbing UVA+UVB light, scavenging ROS, or inhibiting mechanism of NFκB activation. Pathway 4 (in circle) also depends on ROS formed under exposure to UVB. The ROS may directly activate the Nrf2-driven mechanism of gene induction for antioxidant and detoxifying enzymes. The ROS may oxidise also tryptophan, and its oxidative derivatives are triggers for AhR machinery activation. AhR activation leads to indirect induction of Nrf2 as well as to induction of metabolic phase I and II enzymes. AhR pathway controls also immune/inflammatory responses through cytokine and growth factor induction. PPPs may affect this pathway by scavenging ROS, triggering/inhibiting AhR, and inducing Nrf2. Pathway 5 (in circle) is connected to Toll-like receptor (green triangle) for bacterial lipopolysaccharide (LPS), NFκB being a major down-stream target for the activated receptor. The PPPs effects reflect their inhibition of NFκB activation.

κB activity, and cytokine release [127]. Important applications of specific PPPs are envisaged for tissue protection in the course of photodynamic therapy (PDT), an emerging treatment of skin tumors and non-tumor hyperproliferative skin disorders such as psoriasis [128].

ADVANTAGES *VERSUS* PROBLEMS IN THE DEVELOPMENT OF PHENYLPROPANOID-BASED ANTI-INFLAMMATORY DRUGS

In spite of the wealth of laboratory data available on the PPPs anti-inflammatory effects observed *in vitro* and on animal models, many issues remain unsolved in view of effective clinical application. First, large scale evidence-based human studies are awaited, on specific compounds tested in specific therapeutic settings. Another correlated crucial issue for clinical application is represented by administration routes. Different models have been used to demonstrate the protective effects of plant PP topical *vs.* systemic application. To prevent tissue damage, oral administration appears preferable, due to the easier intestinal *versus* cutaneous absorption, and the lower risk of auto-oxidation of the phenol moiety. At present though, in spite of numerous *in vitro* studies demonstrating free radical scavenging and anti-inflammatory properties of plant PPPs, their low bioavailability and the poor contribution to the total antioxidant capacity of plasma renders radical-scavenging properties *in vivo* still uncertain. Conversely, PPPs administrated topically are prone to quick reaction with atmospheric oxygen and other environmental oxidants, and excessively fast inactivation by cutaneous or microbial redox enzymatic systems [43]. A promising perspective for *in vivo* effects is indeed envisaged in the administration to skin through appropriate

stabilizing delivery means [129]. Another important issue is an extremely fast metabolism of PPPs in human organism due to multiple metabolic pathways evolved to eliminate plant-derived toxins [56]. Peculiar PPPs interaction with drug metabolic enzymes may also seriously affect normal drug pharmacokinetics. Several serious adverse (pro-inflammatory, allergic, and toxic) reactions may occur upon PPPs administration to sensitive human skin, mainly, due to controversial effects (anti- *versus* pro-oxidant) of PPPs towards redox processes and towards inflammatory responses in keratinocytes (anti- *versus* pro-inflammatory).

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CONFLICT OF INTEREST

The authors do not have any conflict of interests

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