European Journal of Medicinal Chemistry xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: www.elsevier.com

Research paper

# Indicaxanthin, a multi-target natural compound from *Opuntia ficus-indica* fruit: From its poly-pharmacological effects to biochemical mechanisms and molecular modelling studies

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ARTICLE INFO

*Article history:*
Received 17 April 2019
Received in revised form 1 July 2019
Accepted 2 July 2019
Available online xxx

*Keywords:*
Indicaxanthin
Multi-target compound
Poly-pharmacology
Antioxidant
Antiinflammatory
Antitumoral
Antiproliferative
Neuromodulator
Molecular modelling

ABSTRACT

Over the latest years phytochemical consumption has been associated to a decreased risk of both the onset and the development of a number of pathological conditions. In this context indicaxanthin, a betalain pigment from *Opuntia ficus-indica* fruit, has been the object of sound research. Explored, at first, for its mere antioxidant potential, Indicaxanthin is now regarded as a redox-active compound able to exert significant poly-pharmacological effects against several targets in a number of experimental conditions both *in vivo* and *in vitro*. This paper aims to provide an overview on the therapeutical effects of indicaxanthin, ranging from the anti-inflammatory to the neuro-modulatory and anti-tumoral ones and favored by its high bioavailability. Moreover, biochemical and molecular modelling investigations are aimed to identify the pharmacological targets the compound is able to interact with and to address the challenging development in the future research.



## 1. Introduction

Indicaxanthin is a betaxanthin belonging to the betalain class compound. This includes vacuole pigments restricted to flowers and fruits of 10 families of Cariophyllalae plants and to a few superior fungi of the genus *Amanita* of the Basidiomycetes [1]. Betalains are water-soluble, cationized, nitrogen-containing molecules the colors of which range from the yellow betaxanthins to the violet-red betacyanins. Specifically, the conjugation of betalamic acid with cyclo-dopa, or its glucosyl derivatives, leads to the synthesis of betacyanins, whereas its condensation with amino acids, or the corresponding amines, leads to betaxanthins or derivatives. (see Fig. 1)

Indicaxanthin, the adduct of betalamic acid with proline, is stable in a pH range from 3 to 5 and once released from its protecting vacuolar compartment is susceptible to several degrading factors such as light, enzymatic reactions, and temperature, this latter being the most relevant for its decomposition. Due to the presence of the conjugated dienes of the 1,7-diazaheptamethine structure, indicaxanthin absorbs in both UV and visible region with a maximum at 482 nm; moreover, the pigment is also able to absorb and emit fluorescence at maximum excitation and emission wavelengths of 463 and 515 nm respectively [2].

### 1.1. Biogenesis, structure elucidation, and synthesis

Despite the fact that indicaxanthin and related compounds are usually extracted from natural sources several synthetic methodologies have been reported in literature, because of their biological properties. 411 naturally occurring betacyanins are derived from either betanidin or its C-15 epimer isobetanidin and differ only in the sugar moiety and/or in the carboxylic acid attached to the sugar by an ester linkage. The structure of betanin was elucidated by Dreiding et al., and the relationship between the betacyanins and betaxanthins was established firmly by chemical interconversions of betanidin and indicaxanthin [3–6].

Betaxanthins and betacyanins are characterized by the same dihydropyridine moiety and can be formally considered as immonium derivatives of betalamic acid. This compound was proposed as an intermediate in the biosynthesis of betacyanins and betaxanthins, which would be formed on condensation with appropriate amino acids or amines, and moreover the suggestion was made that it might originate from 3,4-dihydroxyphenylalanine (dopa) (Scheme 1). Although

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https://doi.org/10.1016/j.ejmech.2019.07.006
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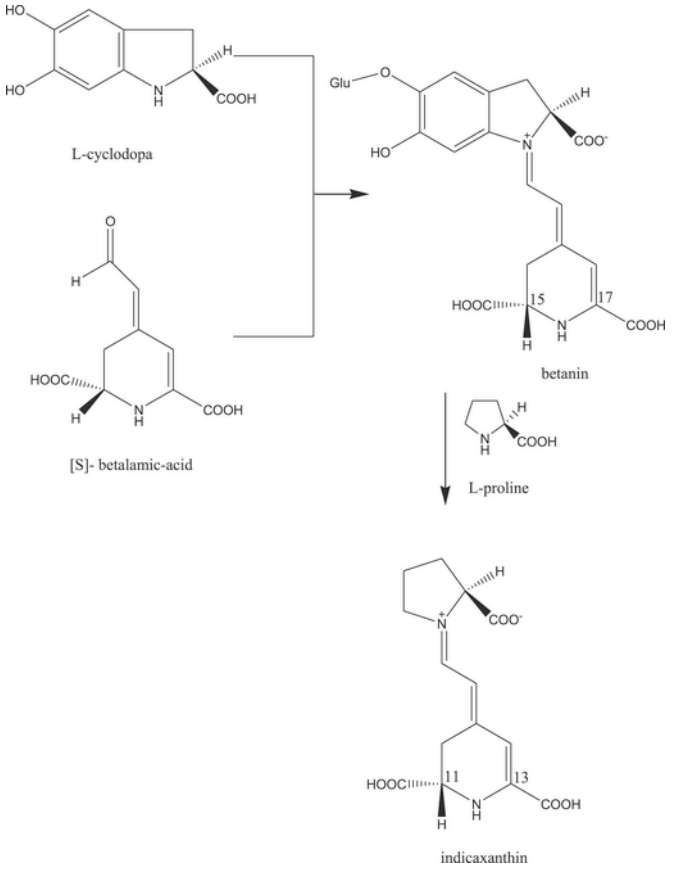



**Scheme 1.** Proposed biosynthesis of Indicaxanthin.

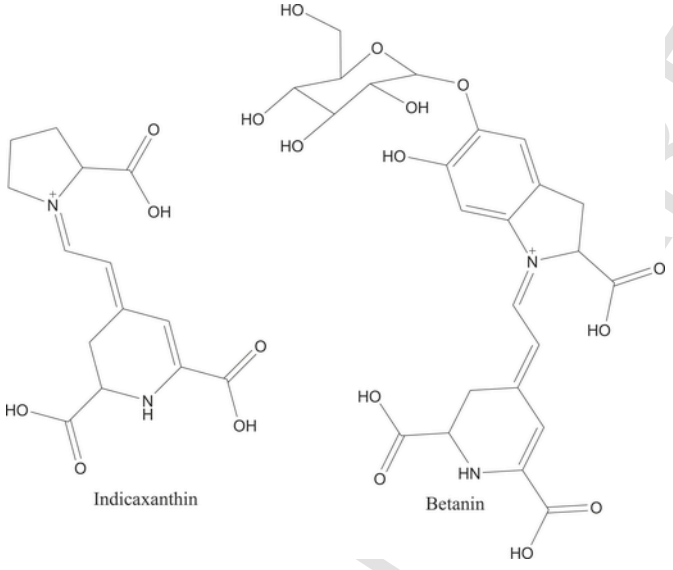



**Fig. 1.** Indicaxanthin and betanin.

betalamic acid had been suspected of being an intermediate in these conversions, it was not until 1971 that it was isolated .

Condensation with L-proline and with synthetic cyclodopa gave indicaxanthin and betanidin, respectively. Betalamic acid was later isolated also from fly agaric (*Amanita muscaria*) along with muscaflavin (Fig. 2) [7,8]. Betanidin proved to be a sensitive compound,

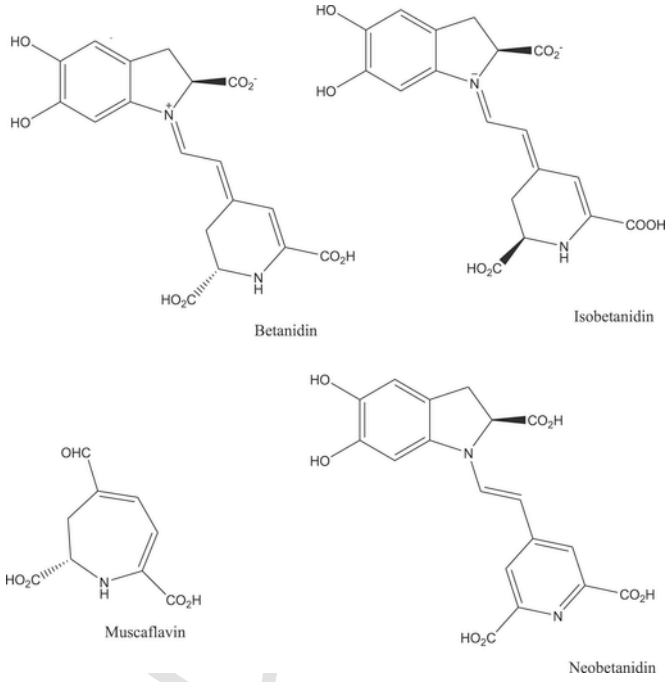
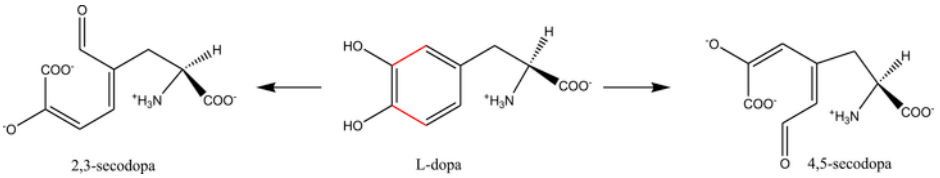



**Fig. 2.** Compounds isolated from fly agaric (*Amanita muscaria*).

and, especially in alkaline medium, it is oxidized easily to the much more stable neobetanidin [3–6]. Due to this instability, synthetic efforts have been directed toward more stable derivatives of both betanidin as well as the dihydroindole and dihydropyridine portions of the molecule. Three syntheses of the so-called cyclodopa moiety, all starting from 3,4-dihydroxy-phenylalanine, have been described [9–11], and stable derivatives of this intermediate are now known.

The biogenesis of betalamic acid was investigated by using labeling experiments. Thus, when 14C-labeled dopa was used in feeding yellow flower buds of *Portulaca grandiflora* labeled betalamic acid was obtained [12]. This labeled acid was converted to 14C-labeled betanin in order to obtain a stable substance recrystallized to a radio-pure sample. Decarboxylation of the radiopure betanin, obtained from the sequence using dopa-1-14C indicated that the labeled carboxyl group of dopa is corresponding to a 14C-carboxyl group in betanin and hence in betalamic acid. The shape of the ORD curve for the naturally occurring betalamic acid was the same as that recorded for a sample of S-betalamic acid obtained by degradation of betanin. Therefore, the hypothesis that betalamic acid is formed *in vitro* by an oxidative cleavage of L-dopa could be supported. Moreover, it is also an intermediate in the biogenesis of other betalains from dopa.

Further studies elucidated the biosynthetic intermediates generated from L-dopa by an enzyme system extracted from *Amanita muscaria* and their spontaneous conversion to muscaflavin and betalamic acid, respectively, and betalains [13]. This enzyme cleaves the C(2)–C(3) and the C(4)–C(5) bond of the aromatic ring of L-dopa to form a mixture of 4,5-secodopa and 2,3-secodopa, two hitherto hypothetical biosynthetic intermediates (Scheme 2). Though isolation of these products was not possible, structural evidence was provided, including spectroscopic data in comparison with known compounds. They cyclize without enzyme catalysis, optimally at pH 4.5–5, to produce muscaflavin and betalamic acid, respectively. Once again the products were identified by direct comparison with authentic samples. Correspondingly, the enzymic conversion of D-dopa to (R)-betalamic acid and its condensation with L-proline produced isoindicaxanthin, which is unknown in nature.




**Scheme 2.** Biosynthetic pathways from L-dopa. The metabolites are shown in the form prevailing in neutral medium.

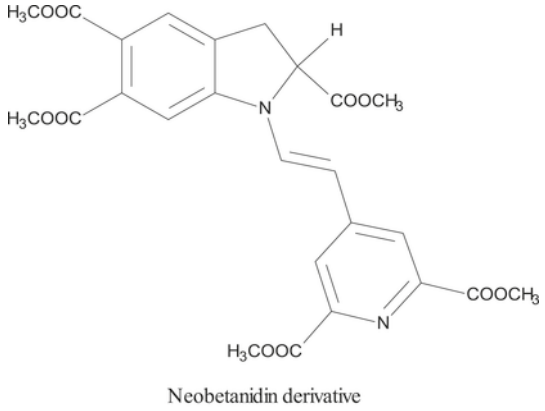



**Fig. 3.** Neobetanidin derivative.

Therefore, this work contributed to clarify the essential steps in the biosynthetic conversion of L-dopa to the betalains.

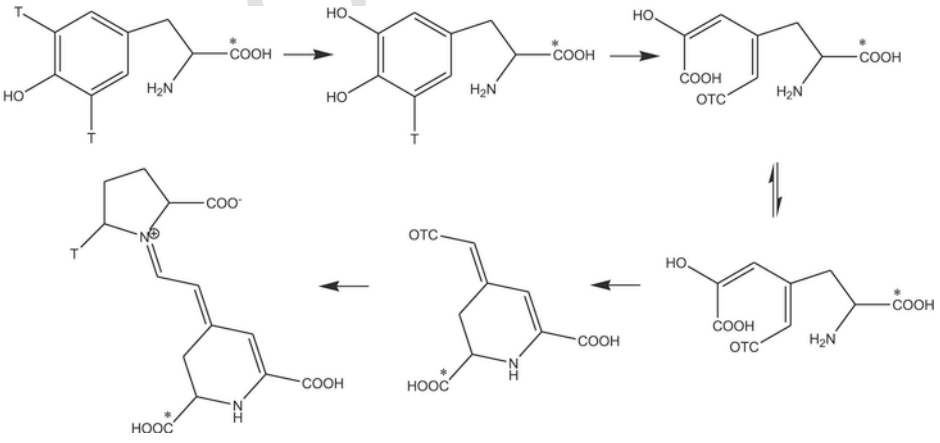
Initially the relationships between betanin and indicaxanthin, and other betalains, were investigated. In fact, when betanin was treated with L-proline, a base exchange took place giving rise to indicaxanthin (Scheme 1) [6]. In this way this yellow pigment was partially synthesized by a method which should be generally applicable for the mutual interconversion of betacyanins and betaxanthins. For example, it was also possible to transform indicaxanthin into betanidin by a base exchange with 5,6-dihydroxy-2,3-dihydroindole-2S-carboxylic acid. The functional fragment common to the betacyanins and the betaxanthins is betalamic acid.

In other studies on the biogenesis of betalains in cactus fruits DL-dopa labeled with 14C in position 1 and 2 was incorporated into betanin which was obtained radiopure after crystallization [14]. The specific activity remained constant after conversion to betanidin and to a neobetanidin derivative (Fig. 3). Reaction of radiobetanin with proline afforded indicaxanthin carrying more than 90% of the radioactivity. Thus, it was possible to demonstrate that dopa is an efficient precursor for betalamic acid but not for cyclodopa. Decarboxylation of radiobetanidin and radioindicaxanthin showed that the carboxyl group of dopa remained a carboxyl group in the biotransformation to betalamic acid. It was concluded that the aromatic ring of dopa is cleaved and that recyclization involving the nitrogen generates the dihydropyridine moiety. Under the same conditions mevalonic acid, aspartic acid, and phenylalanine showed low incorporations. Studies with beet seedlings and DL-dopa and DL-tyrosine, labeled with [14C] in position 1, afforded similar results but with low incorporations.

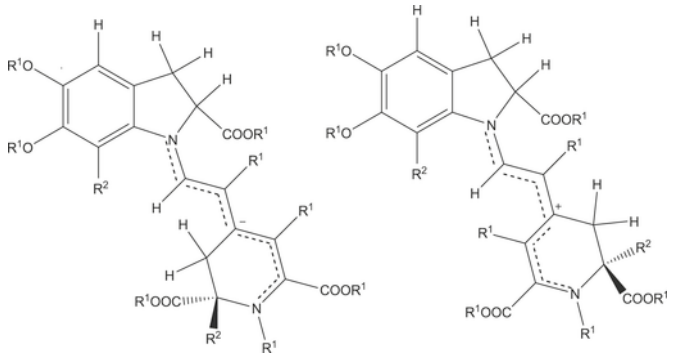
During the elucidation of the biosynthesis of these pigments, feeding experiments in beets with tyrosine-1-14C, 15N or tyrosine-2-14C, 15N labeled demonstrated intact incorporation of the amino acid, including the amino nitrogen, into cyclodopa and the betalamic acid moiety of betanin [15]. However, phenylalanine was not incorporated into betanin. Degradation of betanin labeled with tyrosine-1-14C indicated that 35.4% of the radioactivity was incorporated into indicaxanthin, whereas label from tyrosine-2-14C was incorporated mainly into 5-hydroxy-6-methoxyindole-2-carboxylic acid (64.5–69.7%) with a smaller amount (24.8–34%) into indicaxanthin. In red and green plants of *Kochia scoparia*, feeding of tyrosine-2-14C indicated that alkaloids were labeled prior to betalain formation. It was also demonstrated that both nitrogen atoms seem to be catalytically active.

The result of the incorporation of doubly labeled (Tritium and 14C) tyrosine into indicaxanthin in the fruits showed that the dihydropyridine moiety of the pigment originates from dopa by the so-called "extradiol cleavage" of the aromatic ring [16]. On the basis of these results, a pathway for the biosynthesis of indicaxanthin was proposed, as outlined in Scheme 3.

Betaxanthins are the conjugation products of betalamic acid with any amino acid (both protein or non-protein) or amine [although only a few have been described as naturally occurring]. Indicaxanthin, the first betaxanthin to be described [3], was extracted from the fruit of



**Scheme 3.** Proposed pathway for the biosynthesis of Indicaxanthin in *Opuntia ficus-indica. T = Tritium, * = 14C.*



**Scheme 4.** E/Z isomers interconversion: a ($R^1$=$R^2$=H); b ($R^1$=D, $R^2$=H); c ($R^1$=H, $R^2$=D).

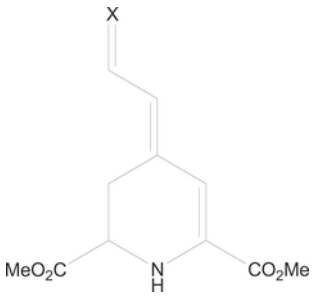
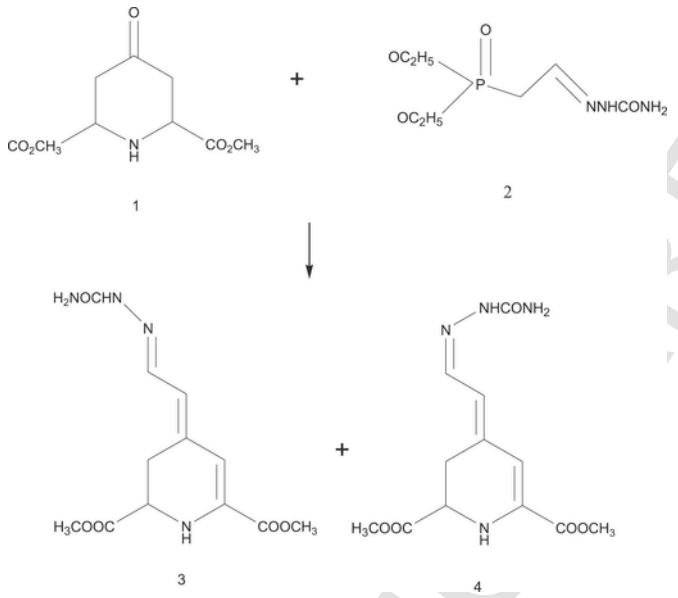


**Fig. 4.** Semicarbazone of betalamic acid (X=$NNHCONH_2$).



**Scheme 5.** cis-4-oxo-2,6-piperidinedicarboxylic acid dimethyl ester (**1**); modified Horner-Wittig reagent (**2**); mixture of semicarbazones of 2,3-dihydrobetalamic acid dimethyl ester (**3** and **4**).

*Opuntia ficus-indica,* and was characterized by a typical absorption band at 485 nm in water. In the paper by Trezzini and Zryd [17], a complete investigation on chromatographic (reverse phase-ion pair HPLC) and spectral properties of the betaxanthins is described.

Few 1H NMR data for betaxanthins have been reported [3,17–22]. Acidification has been considered a prerequisite for appropriate signal detection during NMR measurement. However due to their inherent liability towards highly acidic conditions, NMR spectroscopy was scarcely reported and was, hitherto, exclusively based on 1H NMR data interpretation.

Unexplained 1H NMR signals of betanidin and indicaxanthin in $CF_3COOH$ are interpreted via deuteration experiments in deuterated trifluoroacetic acid [20]. The authors confirmed the 1,7-diazaheptamethinium chromophore of these pigments. An E-Z stereoisomerism at 1 of the partial double bonds (mixture ratio 75:25) was observed. However, the E-Z interconversion is so fast that these isomers cannot be separated; the structures of deuterated and/or protonated forms (a-c) are shown in Scheme 4.

Applying only slightly acidic conditions, Stintzing et al. were able to report the first 13C NMR data of two betaxanthins, i.e., indicaxanthin and of miraxanthin [23]. NOESY and NOE experiments [in acidified (0.01% TFA) $D_2O/H_2O$ 1:4 (v/v)] allowed to assign all the signals. Strong solvent dependencies on the formation of the preferred stereoisomers as well as the presence of dynamic equilibria of at least four detectable stereoisomers of indicaxanthin and miraxanthin in aqueous solutions were clearly observed.

Most of the studies which approached synthetic methodologies started from what was discovered during the analysis of the biosynthetic pathways leading to betalain derivatives.

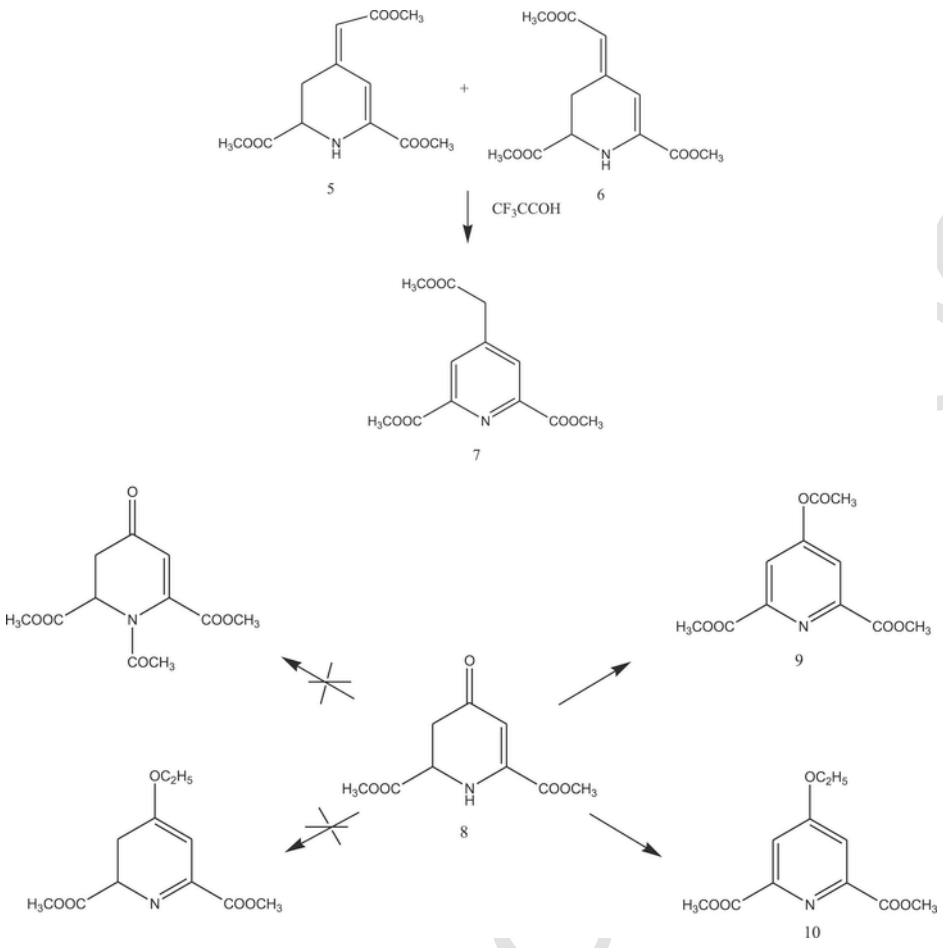
Thus, the first total synthesis of betalamic acid and betanidin was reported by Hermann and Dreiding [24].

The key intermediate was betalamic acid in the form of its dimethyl ester semicarbazone (Fig. 4), which was transformed into the corresponding dimethyl ester of indicaxanthin (if treated with L-proline) or into betanidin trimethyl ester (if treated with L-cyclodopa methyl ester). Of course betadin was obtained upon hydrolysis.

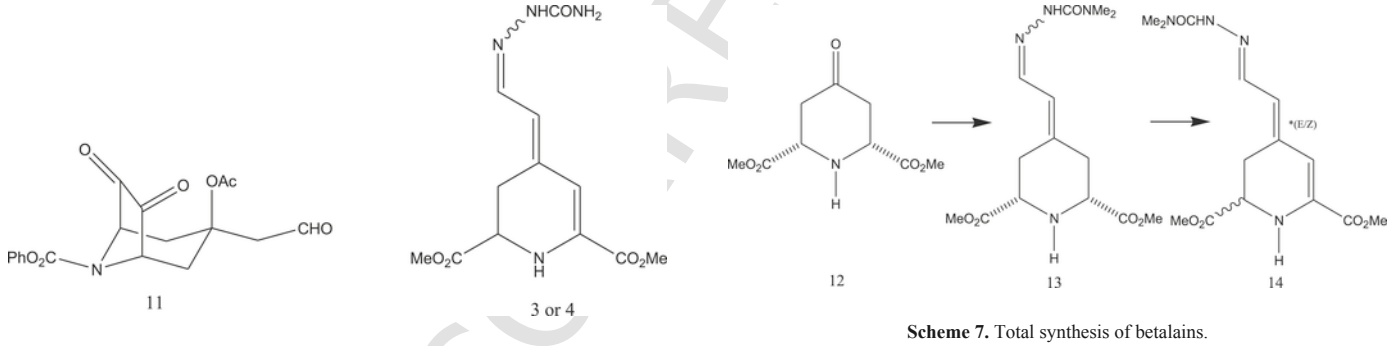
Two years later the same authors reported the use of *cis*-4-oxo-2,6-piperidinedicarboxylic acid dimethyl ester (**1**) as precursor of indicaxanthin and betanidin [25]. In fact, derivative **1** was converted into the mixture of semicarbazones **3** and **4** of 2,3-dihydrobetalamic acid dimethyl ester in 44% yield, by using a modified Horner-Wittig reagent **2**. Oxidation of **3**/**4** afforded 41% of a mixture of stereoisomers of the corresponding betalamic acid derivative, key intermediate for the synthesis of betalain pigments. To this respect the utility of this compound was demonstrated on a small scale by its conversion to the dimethyl ester of indicaxanthin (11%) and to the trimethyl ester of betanidin (87%). Hydrolysis of ester functions gave betanidin (Scheme 5).

In the same paper also the synthesis of the trimethyl ester of an oxidized form of betalamic acid **7** was described as well as model condensation reactions on the carbonyl group of cyclohexanone, *cis*-4-oxo-2,6-diphenylpiperidine, and its N-formyl derivative. Reaction of 4-oxo-1,2,3,4-tetrahydro-2,6-pyridinedicarboxylic-acid dimethyl ester (**8**) with acetic anhydride or with triethyloxonium tetrafluoroborate resulted in O-acylation or in O-alkylation along with dehydrogenative aromatization to yield the derivatives of chelidamic acid **9** or **10**, respectively (Scheme 6).

Later on, a new Horner-Emmons reagent was used to create the unsaturated aldehyde side chain, starting with 4-oxo-2,6-cis-piperidinedicarboxylic acid dimethyl ester. The second cyclic double bond was introduced by Pfitzner-Moffat oxidation. The known instability of betalamic acid to oxidizing agents prompted research groups to undertake other synthetic pathways in which unwanted aromatizations could be avoided until the final product has been isolated. For this reason, the second olefinic bond was introduced by an elimination, rather than an oxidation reaction. In the paper by Buchi, Fliri, and Shapiro [26], in details, it is described a procedure in which the dihydropyridine framework used in the first total synthesis was replaced by a tropane template.



**Scheme 6.** Synthesis of the trimethyl ester of an oxidized form of betalamic acid **7**; derivatives of chelidamic acid **9** or **10**.



**Scheme 7.** Total synthesis of betalains.

**Fig. 5.** Diketo aldehyde (**11**); dimethyl betalamate, characterized as a crystalline semicarbazone (**3** or **4**) of unknown stereochemistry.

A long sequence of reaction, reported in details in the original work, allowed to prepare the diketo aldehyde **11** (Fig. 5).

Treatment of this last with lead tetraacetate in methanol/benzene afforded a dimethyl ester which, upon chromatography over silica gel, lost both acetic and benzoic acid to give dimethyl betalamate, characterized as a crystalline semicarbazone **3** or **4** of unknown stereochemistry (Fig. 5). However, this last compound was converted to indicaxanthin and betanidin in the usual way.

Total synthesis of betalains was achieved according to Scheme 7 [27]. Formyl-olefination of *cis*-4-oxo-2,6-piperidinedicarboxylate dimethyl ester with a Horner-Wittig reagent (of type **2**, dimethylamino substituted) led to a piperidylidene acetaldehyde derivative of type **12**, which was dehydrogenated, by treatment with $Me_3COCl$ and then $Et_3N$, to give compound **13**. Exchanging the semicarbazone moiety with an (S)-cyclodopa derivative, with (S)-proline, and with indoline led to the didehydro-betanidin, indicaxanthin, and racemic indo-betalaine, respectively. The latter, a new, relatively stable betalain, was hydrolyzed and esterified to the corresponding racemic ester of betalamic acid. Under the influence of $NH_3$/MeOH, the dimethyl ester of racemic indo-betalain was dehydrogenated spontaneously to indo-neobetalaine dimethyl ester. Synthetic betanidin consisted of a 4:6 mixture of the (natural) (2S,15S)- and the (2S,15R)-isomer and both of a 75:25 mixture of the E and the Z isomer. Synthetic indicaxanthin and the indo-betalaine consisted of a 65:35 and a 70:30 mixture of the E and the Z isomers, respectively. All isomers are rapidly interconvertible, and temperature-dependent 1H-NMR-measurements of

**Table 1**
Summary of the biochemical and pharmacological effects of indicaxanthin.

| Properties/Effect | System/Model | Ref |
|---|---|---|
| Reducing potential | Cyclic Voltammetry | 28 |
| Radical scavenging | Solution, Liposomes | 28–30 |
| Amphiphilicity | Liposomes, Lipoproteins, Membranes, Erythrocytes | 31–35 |
| Anti-oxidative | Solutions, Vesicles, Liposomes, Lipoproteins, Erythrocytes | 31–35 |
| Anti-inflammatory | Endothelial cells, Macrophages, Colon cells, Rat | 41,42,44,47,49,50 |
| Anti-proliferative | Melanoma cells, Rat, Colorectal Carcinoma cells | 52,56,57 |
| Permeability to the Brain-Blood Barrier | Rat | 58 |
| Neuromodulatory | Rat, neurons | 58,59 |
| Bioavailability | Human, Colon cells | 60,65 |

**Table 2**
Target identified by Reverse Docking methodology, with related biological pathways, and calculated binding data.

| Target protein | [a]$\Delta G_{bind}$ | Pathway |
|---|---|---|
| ITP3K | −50.460 | Decrease of the release of Neurotransmitters (Synaptic plasticity) |
| GCPII | −100.903 | Glutamate release (Glutamatergic transmission) |
| LTA4H | −48.457 | Inflammation associated carcinogenesis |
| HPSP | −52.715 | Increase of synthesis of D–serine, co-agonist for NMDA receptor in the glycine binding site (Glutamatergic transmission) |
| PDE4D | −43.116 | cAMP Hydrolysis, PKA Activator (Mnemonic processes and intestine contractility) |
| GluA3 | −55.390 | Glutamatergic transmission |
| GluA2 | −55.262 | Glutamatergic transmission |
| GluK1 | −59.335 | Glutamatergic and GABAergic transmission |

[a] Free energies (kcal/mol) (best value predicted by MM-GBSA calculations).

racemic indo-betalain established $\Delta G = 84.7$ kJ/mol for the E-to-Z conversion.

## 2. Poly-pharmacological effects of indicaxanthin and biochemical mechanisms

### *2.1. Reducing and antioxidative properties*

The reducing potential of indicaxanthin has been investigated by cyclic voltammetry: two peaks of 611 and 895 mV have been calculated from the differential pulse voltammogram [28]. In line with this, the phytochemical has been shown to act as an effective scavenger of a number of radicals and reactive oxygen and nitrogen species (RONS), ranging from the cation radical of ABTS and the radical of DPPH to the HOCl and the redox intermediates of the peroxidase cycle of MPO [28–30].

From a physical-chemical perspective indicaxanthin is not a mere water-soluble hydrophilic pigment. Indeed, thanks to its charged and ionizable groups, as well as lipophilic moieties, it may behave as amphiphilic-like compound at physiological pH [31]. The phytochemical has been demonstrated to bind to large unilamellar dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) liposomes, human lipoproteins, and biological membranes [32–35]. Moreover, spectrophotometric and kinetic studies on the solubilization site of indicaxanthin into DPPC and DMPC vesicles indicate that indicaxanthin is not located within the hydrophobic core of the vesicles but in the palisade domain of the bilayer, at the interface between hydrophobic core and hydrophilic head groups [31].

Thanks to its reducing potential and its amphiphilicity indicaxanthin can react with a wide range of aqueous lipoperoxyl radicals adsorbed onto vesicular-like surfaces [29]. In line with this, the phytochemical has been demonstrated to exert significative antioxidative effects in a number of experimental set-up *in vitro* and has been often regarded as a cationized antioxidant. In this regard, indicaxanthin exhibits a classical chain-breaking antioxidant behavior when included in methanol solution of methyl linoleate (LAME) submitted to oxidation by 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN). In this system the efficacy of the molecule as a lipoperoxyl radical-scavenger is comparable to that of a lipid antioxidant such as vitamin E, with a stoichiometric factor approaching 2 and an inhibition constant of the same order as that reported for α-tocopherol under similar conditions. Indicaxanthin is quite effective in preventing lipid peroxidation, also when incorporated into liposomal bilayers of soybean phosphatidylcholine submitted to aqueous radicals from 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH). However, in this system, its activity was not consistent with the antioxidant effects observed in solution, suggesting a mechanism more complex than that of a classical chain-breaking lipoperoxyl radical scavenger. Rather, it appeared to depend on its interactions with the phospholipid bilayer and to be enhanced by the concurrent action of α-tocopherol (when added to liposomes) with whom the phytochemical was also able to cooperate and to establish reciprocal protective interactions [29].

Moving towards more complex amphipathic systems such as human LDL, indicaxanthin has been demonstrated to be incorporated within them with a maximum binding of $0.52 \pm 0.08$ nmoles of indicaxanthin per mg LDL protein. Moreover, the incorporated indicaxanthin was able to protect LDL to copper-induced oxidation as assessed by the elongation of the lag phase. Interestingly enough, indicaxanthin interacted with vitamin E, prevented its consumption at the beginning of LDL oxidation and prolonged the time of its utilization, significantly enhancing LDL protection [33].

Remarkably, indicaxanthin has been shown to exert its antioxidative effects, even in cellular environments such as red blood cells (RBC) either from healthy subjects or from β-thalassemia patients [35]. In this regard, the phytochemical is able to incorporate within RBC, to protect them from cumene hydroperoxide-induced hemolysis, preventing lipid and haemoglobin oxidation and counteracting vitamin E and GSH depletion. Interestingly, the antioxidative effects of indicaxanthin on oxoferryl haemoglobin are in line with the previously cited capacity of indicaxanthin to scavenge the perferryl intermediates of the MPO catalytic cycle [35]. This result appears of particular interest when considering the potential use of this phytochemical to protect the redox machinery of thalassemic RBC usually worn out by the oxidative stress that is a hallmark of this haemolytic disorder.

### *2.2. Anti-inflammatory properties*

The role of RONS in the disruption of cellular architecture is well established and is believed to be mediated by the oxidative and nitrosative alterations of cellular biomolecules [36]. On the other hand, finely tuned alterations of the endocellular redox state are now strongly believed to be responsible for the activations of specific, redox-dependent signalling pathways fostering and leading to cellular activation and dysfunction. In line with these data, inflammatory, oncological, cardiometabolic and neurodegenerative pathologies are now believed to be strongly connected with a disruption of the cellular redox tune [37,38].

Inflammation is an adaptive response triggered by noxious stimuli and conditions, such as infection and tissue injury. At a very basic

level, it involves recruitment of both blood components and cells of the adaptive and innate immunity system to the site of infection or injury. This process is coordinated by a large range of biochemical mediators, and results in the inflammatory exudate. The acute phase of an inflammatory response successfully ends up in the elimination of the insult, followed by a resolution phase, mainly mediated by tissue-resident and recruited macrophages [39].

Among the innate immunity cells, the endothelial ones (EC) do play a crucial role in most of the inflammatory-related dysfunctions and therefore constitute an ideal target for the research and the development of anti-inflammatory drugs [40].

In this regard, indicaxanthin has been demonstrated to protect EC from both TNF-α- and oxidized LDL (oxLDL)-induced dysfunction. In these systems, the phytochemical modulated specific redox-dependent signalling pathways responsible for EC activation. Indeed when stimulated with 1 ng/ml TNF-α, HUVEC pre-treated with indicaxanthin at 5 μM showed a reduction of cytokine-induced ICAM-1 over-expression (−15%) [41]. Similarly, when stimulated with 100 μg/ml oxLDL, EC pre-treated with indicaxanthin in the range between 5 and 20 μM, showed a concentration-dependent reduction of oxLDL-induced ICAM-1, VCAM-1 and ELAM-1 over-expression (both at mRNA and protein levels). At the same time, oxLDL-induced ABC-A1 down-regulation was prevented. From a mechanistic perspective, these effects were correlated with a reduction of endocellular RONS levels that in turn counteracted the redox-dependent NF-κB activation [42]. Overall, these anti-inflammatory effects of indicaxanthin in EC do require further pharmacological studies to deeper investigate its potential against inflammatory and cardiometabolic conditions such as atherosclerosis where oxLDL/cytokine-mediated EC damage plays a crucial role.

Within the innate immunity, macrophages are crucial effectors cells able to synthesize relatively high amounts of RONS as a part of their molecular defence machinery against pathogens and tumor cells [43]. On the other hand, the same cells finely modulate their redox milieu to control metabolic responses during inflammation, in order to foster the switching from the acute phase to its resolution, thus avoiding chronicity. The redox-dependent metabolism of arachidonic acid (AA), with expression of inducible cyclo-oxygenase-2 (COX-2), its downstream enzymes PGE2 synthase-1 (PGES-1) and PGD2 synthase (PGDS), the release of the pro-inflammatory prostaglandin E2 (PGE2), the anti-inflammatory PGD2 and its derivative 15-deoxy PGJ2 (15D-PGJ2), are central in the inflammatory process [44].

In contrast to the majority of drugs and nutraceuticals that commonly exert anti-inflammatory effects by down-regulating COX-2 expression that in turn shut down the biosynthesis of all PG, indicaxanthin counteracts macrophages activation and elicits anti-inflammatory effect in a completely different fashion. The molecule has been demonstrated to divert the metabolic axis of PG biosynthesis from PGE2 to PGD2. Indeed, LPS-activated RAW 264.7 macrophages preincubated with indicaxanthin in a range between 50 and 100 μM, showed a significant COX-2 overexpression and at the same time a PGES-1 down-regulation and PGDS up-regulation both at protein and mRNA level [44]. As a consequence, COX-2 reaction product (PGH2) cannot react with PGES-1 and conversely becomes substrate for PGDS; this leads to a decrease of PGE2 release and an increase of PGD2 synthesis that is subsequently metabolized to PGJ2. The mechanism for such a metabolic rewiring is NF-κB-dependent and passes through the modulation of macrophages redox-state i.e. a prooxidant activity of indicaxanthin through lipid peroxide production. Rather than simply arresting the inflammatory reaction, the molecule appears to induce a cellular reprogramming towards the anti-inflammatory state and these effects could be of interest to project a novel class of anti-inflammatory compounds [45,46].

Interestingly enough, not only modulates indicaxanthin the activation of LPS-stimulated macrophages, but also protects these cells from 7-ketocholesterol (7-KC)-induced apoptosis, a key event in the development of atherosclerosis [47]. 7-KC-stimulated macrophages pre-treated with indicaxanthin in the range between 0.1 and 2.5 μM, showed a significant reduction of 7-KC-induced apoptosis. The mechanism for these protective effects where mediated by:

i) the modulation of endocellular macrophage redox state and $Ca^{++}$ homeostasis,
ii) the inhibition of NF-κB activation and NOX-4 activity and expression,
iii) the protection of mitochondria from oxidative damage.

Noteworthy, the anti-inflammatory potential of indicaxanthin is not confined to EC and macrophages but it is also evident in other cellular districts such as epithelial cells. Stimulation of these cells by IL-1β is widely acknowledged as an *in vitro* model of chronic inflammatory bowel disease, as this cytokine is known to play a major role in the initiation and amplification of the inflammatory reaction in such a pathology [48]. Preincubation of IL-1β -activated Caco-2 cells with indicaxanthin in the range between 1 and 25 μM, prevented the release of the pro-inflammatory cytokines IL-6 and IL-8, PGE2 and NO, the endocellular production of RONS and the decrease of thiols in a concentration-dependent manner. Moreover, co-incubation of the cells with indicaxanthin and IL-1β also prevented the IL-1β-induced increase of epithelial permeability. These anti-inflammatory effects were mediated by the inhibition of NOX-1, COX-2, iNOS over-expression and NF-κB activation [49].

Finally, the above-reported anti-inflammatory effects, exerted *in vitro* by indicaxanthin, were paralleled *in vivo* in a reproducible model of acute inflammation, i.e. the carrageenin pleurisy, widely considered a gold standard tool to evaluate anti-inflammatory drugs [50,51]. Administration of indicaxanthin (0.5, 1, 2 μmol/kg) per os in rat challenged with λ-carrageenin, time- and dose-dependently, reduced the exudate volume and the number of leukocytes recruited in the pleural cavity. Pre-treatment with indicaxanthin at 2 μmol/kg inhibited the carrageenin-induced release of PGE2, NO, IL-1β, TNF-α in the exudate and caused a decrease of IL-1β, TNF-α, iNOS, and COX-2 mRNA as well as iNOS and COX-2 protein expression in the recruited leukocytes. These effects were mediated by the time- and dose-dependent inhibition of NF-κB activation [50].

### *2.3. Antiproliferative and antitumoral effects*

Cancer is a growing health problem worldwide and according to the International Agency for Research on Cancer, 14.1 million new cancer cases and 8.2 million cancer deaths worldwide have been reported [52].

The causative link between inflammation and melanoma has accurately been investigated in recent years. In this regard, reciprocal interactions between melanoma and immune cells in a pro-inflammatory microenvironment provide a source of phenotypic heterogeneity that drives therapy resistance and metastasis [53–55]. In line with these interconnections and the anti-oxidative and anti-inflammatory properties of indicaxanthin, the molecule has been recently demonstrated to exert significant antiproliferative effects on melanoma cell lines and behave as an antitumoral compound against melanoma *in vivo*. Specifically, indicaxanthin (in the range between 50 and 200 μM) was able to induce apoptosis of human melanoma cells (A375, Sk-Mel-28, MALME, B16/F10) through the inhibition of

both NF-κB pathway and the downstream over-expression of two anti-apoptotic proteins, B cell lymphoma gene-2 (Bcl-2) and FLICE-inhibitory protein (c-FLIP), whose transcription is modulated by NF-κB.

Interestingly enough, these *in vitro* data were paralleled *in vivo* in a murine model of melanoma obtained by the subcutaneous injection of B16/F10 murine melanoma cells in C57BL/6 mice. In this experimental system, indicaxanthin administered *per os* at 3.2 mg/kg induced, 14 days after tumor implantation, a reduction of both tumor volume (86%) and weight (83%). Moreover, in the plasma of indicaxanthin-treated mice a significant reduction of CXCL1 levels by 42% was observed as compared to control mice. CXCL1 is a chemokine belonging to the CXC chemokine subfamily and has been associated with metastatic melanoma since it fosters the recruitment of tumor promoting myeloid cells into the tumor and enhances angiogenesis.

It is important to underline how the complexity and aggressiveness of melanoma make it hardly to be controlled with just one pharmacological agent. Along these lines indicaxanthin maybe proposed as a novel therapeutical agent to be further explored in more complex studies in combo therapy, i.e. with other therapeutical agents targeting different check points of melanoma development.

Relevantly, besides melanoma cells, indicaxanthin has been shown to exert antiproliferative effects against other cancer cells such as human colon cancer cells (Caco-2). When preincubated in the range between 1 and 250 μM, the molecule significantly counteracts cell proliferation with an $IC_{50}$ of 115 μM and without toxicity for normal colon cells. From a mechanistic perspective, these effects were not mediated by a modulation of the endocellular oxidative stress by indicaxanthin. Rather, the molecule was able to induce epigenomic modifications such as de-methylation of the tumor suppressor p16INK4a gene promoter, reactivation of the silenced mRNA expression and accumulation of p16INK4a, a major controller of cell cycle. As a consequence, decrease of hyper-phosphorylated, in favor of the hypophosphorylated retinoblastoma was observed, with unaltered level of the cyclin-dependent kinase CDK4. Cell cycle showed arrest in the G2/M-phase [56]. Moreover, indicaxanthin has shown anti-proliferative activity in other colorectal cell lines such as LoVo1, HT-29, HCT116, and DLD1. The mechanism involved demethylation in the promoters of some methylation-silenced onco-suppressor genes supporting colorectal carcinogenesis (p16INK4a, GATA4, and ESR1) leaving unchanged others which were basally hypermethylated (SFRP1 and HPP1). Indicaxanthin also increased the expression of genes involved in DNA demethylation [57].

### *2.4. Neuromodulatory effects*

The neuropharmacological effects of many drugs and nutraceuticals are crucially dependent from their ability to cross the blood brain barrier (BBB) that usually shields brain from the majority of xenobiotics [58]. In line with this, only quite a few phytochemicals have been reported to exert neuroprotective effects. Indeed, among all flavonoids, flavanols and anthocyanins are the only ones able to cross the BBB, accumulate within several regions of the brain, and modulate memory and learning processes. However, because of limited bioavailability, these molecules are poorly absorbed in the brain, especially anthocyanins.

Conversely and interestingly enough, indicaxanthin has been recently demonstrated to cross the BBB and accumulate within the brain in rat [58]. After a single oral administration of 2 μmol/kg body weight, the pigment was detectable after 60 min, and a peak of 20 ± 2.4 ng/g of fresh brain was reached 2.5 h after administration. Indicaxanthin disappeared from brain at 4 h and, log transformation of its amount within 2.5–4 h after ingestion, indicated that the disposal followed first-order kinetics. The calculated half-life was 0.82 ± 0.07 h. As previously reported [50], oral administration of 2 μmol/kg resulted in a 0.20 μM indicaxanthin plasma peak at 2.5 h in rat, with total disappearance within 4 h with first-order kinetics. Comparing present and previous data and considering a mean rat blood volume of 16 mL, it can be calculated that the amount of the molecule in the brain at 2.5 h (a time point reflecting an equilibrium of distribution already reached), is approximately 2% of that absorbed.

Interestingly, once crossed the BBB, indicaxanthin distributes in rat brain areas and remains unmodified at levels comparable or even greater than those shown by other phytochemicals [59]. Noteworthy, while some nutraceuticals distribute within the brain homogeneously, indicaxanthin gains a specific access to selected brain areas, being present in different amount in cortex, hippocampus, diencephalon, brainstem, cerebellum, but not in the striatopallidal complex. Indeed, oral administration of the molecule at 2 mmol/kg resulted, after 1 h, in a maximum and minimum amount in cerebellum and cortex (0.09 ± 0.002) and (0.05 ± 0.002) ng/mg of fresh tissue, respectively. While indicaxanthin peculiar ability to cross BBB may be due to its amphiphilicity, high bioavailability [60] and affinity to lipid membranes, a more complex scenario may be envisaged to explain the selective accumulation of the pigment in some brain areas and the exclusion from others such as the striato-pallidal complex. From an anatomical perspective, the discrepancy could be ascribed to the peculiar structural features of this subcortical region belonging to the basal ganglia; in particular, the presence of different fiber bundles, encapsulating the striatum and pallidum, could not allow indicaxanthin accumulation as easily as in the rest of the brain.

Interestingly enough, not only indicaxanthin distributes within selected brain areas, but also modulates the bioelectric activity of neurons *in vivo* [58,59].

Indeed, when administered in a range between 0.085 ng and 0.34 ng per hippocampal rat neuron, the phytochemical dose-dependently modulated the rate of discharge of spontaneously active neurons of the hippocampus, with reduction of the discharge and related changes of latency and duration of the effect. Moreover, indicaxanthin at 0.34 ng/neuron showed inhibitory effects on glutamate-induced excitation, indicating activity at the level of glutamatergic synapses and the ability to affect glutamatergic transmission [58].

In line with that, the bioelectric activity of other neurons belonging to different brain areas is also modulated by indicaxanthin, mainly with dose-related responses [59]. A predominating inhibitory effect, in all the above-mentioned brain areas, has been observed, suggesting that the phytochemical may be envisaged as a new lead-compound able to exert beneficial effects by reducing neuronal excitability and act as a functional brake in the context of excitatory circuits, under physiological conditions. This may be relevant in those neurological pathologies, such as neurodegenerative conditions or complex cognitive brain processes, where a dysfunction of neuronal excitability plays a crucial role.

Thus, as highlighted in the above paragraphs, indicaxanthin exerts numerous effects on several systems, and different biochemical mechanisms have been suggested. Main properties and related models are listed in Table 1.

### *2.5. Computational studies*

Computational and molecular modelling methods have been also used in the case of indicaxanthin (and related compounds) although not to a large extent.

For example, a theoretical determination of the pKa values of betalamic acid and indicaxanthin was related to the free radical scavenger capacity of the compound [61]. It is well acknowledged that the antioxidant power of betalains was strictly related to the dissociation rate of the acid moieties present in all the molecules of this family of phytochemicals. Only the pKa values of betanin have been determined experimentally. Recently, it was evidenced as the acid dissociation, at different environmental pHs, affects its electron-donating capacity, and therefore its free radical scavenging power.

To achieve the theoretical pKa prediction for organic substances two major types of approach have been used: empirical methods and quantum chemical methods. The empirical methods can be further divided into three groups:

1) linear free energy relationships (LFER) methods utilizing the empirical relations of Hammett and Taft,
2) quantitative structure–property relationships (QSPR), methods correlating calculated structural descriptors with pKa values, and
3) methods searching of similar structures in database of molecules with known measured pKa values.

The empirical methods can be realised with high speed, whereas quantum chemical methods, supposed to have higher accuracy, are more time demanding. All these approaches are much more time-consuming than empirical ones. Tutone et al., in their paper, compared experimental versus predicted pKas of a series of phytochemical acids by means of three different methods, two empirical and one quantum chemical with the aim to obtain the most reliable pKa values for betalamic acid. Marvin, Epik, Jaguar Programs were used and the obtained results were in good agreement with literature reports [62,63], confirming Marvin and Epik as the best predictors between the three methods exploited.

It has to be underlined that in this case a particular challenge must be achieved because pKa predictions methods for compounds that contain multiple titratable groups in which corresponding pKas are similar in magnitude are very difficult. The case of betalamic acid drastically complicates the titrations, and experimental values are an average of several simultaneous protonation ''events.''

As observed *in silico*, at such pH values, the di-anionic microspecies predominant in solution, which, exploiting the considerable electron-donating ability, is able to reduce in exhaustive manner the initial concentration of the radical colorant. These observations were further confirmed by the energetic calculation of HOMO and LUMO for the undissociated and dissociated predominant microspecies of Betalamic Acid. In fact, these calculations showed that the totally dissociated microspecies, present in major percentage at pH = 5.5, had the higher value of HOMO energy and the best electron-donating power over the all possible ones. Thus the quantum mechanical DFT calculation gave reliable prediction of the pKas of Betalamic Acid. In conclusion the obtained results by means of these computational approaches are consistent (pKa values in the range of 2.82–3.43, and 4.16–5.18, respectively, for the C10 and the C7) with the experimental findings reported by Gandía-Herrero et al. [64].

Other attempts to determine *in silico* the pKa values and other physicochemical parameters of indicaxanthin have been performed, with the aim to justify the *trans*-epithelial transport across Caco-2 cell monolayers [65].

The solubilization site of indicaxanthin in lipid bilayers was investigated by the kinetics of its oxidation by peroxyl radicals in water and in aqueous/L-α-dipalmitoyl-phosphatidylcholine (DPPC) vesicles pH 7.4, at 37.0 and 48.0 °C i.e. in a gel-like and a crystal liquid-like bilayer state, respectively [31]. Collectively, the results showed that, under these conditions, the binding constants calculated for indicaxanthin are quite similar. Therefore the compound is considered to be partitioned in a region of the DPPC bilayer, whose state is not affected by temperature changes, the so-called "palisade domain". This membrane portion, laying between the hydrophilic head-groups and the hydrophobic core, seems to be an ideal solubilization site for molecules such as indicaxanthin, sharing hydrophilic as well as lipophilic moieties. The time-dependent absorbance decay, matched with a successful simulation of the reaction kinetic mechanism by Gepasi software, supported a multistep pathway. Computer assisted analysis allowed calculation of the rate constants associated with the reactions involved, the values of which decreased with increasing DPPC concentration. The binding constant calculated according to a pseudo two-phase distribution model did not vary with the physicochemical state of the vesicle, indicating location of indicaxanthin in a region whose state is not affected by temperature changes, at the interface between hydrophobic core and hydrophilic head groups. The simulated reaction model matching the experimental data included an intramolecular arrangement of indicaxanthin. Betaxanthins are known to undergo modifications such as decarboxylation and isomerization [2], however decarboxylated products from indicaxanthin have never been isolated [66]. The molecule instead is known to be prone to C-11 isomerization [2].

*Ab initio* S0 state calculations (using MP2 and DFT methods) and also excited-state calculations allowed to complement experimental data [67]. At the ADC(2) and TD-DFT levels of theory it was possible to have insight into the mechanism of the excited state deactivation through the conical intersection region. The authors suggested that most probably the excited-state deactivation process is initiated by the rotation about the CN double bond, present in the chain linking the electron-donor catechol to the electron-acceptor dihydropyridine moiety. It can be followed by collective geometry changes involving a large rotation about the C=C bond. This mechanism seems to be universal for different betaxanthin dyes present in plants, and plays a photoprotective role.

The discovery of the indicaxanthin physiological targets plays an important role in understanding the biochemical mechanism. *In silico* molecular modelling of indicaxanthin with N-methyl-D-aspartate receptor (NMDAR), the most representative of the glutamate receptor family in hippocampus, suggested this receptor as a possible target [58]. The docking poses of the indicaxanthin with GluN1−GluN2A have been compared with the obtained poses of two selective competitive antagonists of the same target: the small D-AP5 (PDBID: 4NF5) and the larger PPDA (PDBID: 4NF6). Indicaxanthin showed a better binding energy, calculated by means of MM-GBSA, in the interaction with GluN2A subunit residues, than the reference compounds. The relevance of the involved residues (K87, R176, G89, S173, Y214, and R121) in the ligand-GluN2A subunit interaction has been explored by computational mutagenesis, replacing each of the aforementioned residues with alanine. All mutations applied were unfavorable, with an increase of $\Delta G_{Bind}$ in a range between 20 and 40 kcal/mol, indicating that K87, R176, G89, S173, Y214, and R121 were fundamental for the indicaxanthin binding with the receptor subunit. However, the interaction with other Glu receptors could not be ruled out at that time.

Later on, an interesting study which combined reverse pharmacophore mapping, reverse docking, and text-based database search was proposed to furnish an insight into possible interactions between indicaxanthin and other CNS neuromodulator receptors, CNS expressed, anti-cancer and anti-inflammatory targets [68]. The reverse pharmacophore mapping method taps into the concept of shape similarity, according to which chemical compounds of similar shape are

likely to have similar biological activity, binding the same target proteins [69,70].

The reverse docking method, in contrast to the traditional molecular docking used to find the ligands of a target protein, allows docking a small molecule into the predefined binding sites of a pool of protein structures. The strategy requires a sufficient number of known three-dimensional structures of proteins. Further molecular dynamics simulations and binding free energy calculation were performed to understand the mechanism of indicaxanthin to modulate the biological targets. The flowchart of this very complex methodology can be seen in details in the original work. It includes various types of docking procedure as well as molecular dynamics simulations and MM-GBSA free energy calculations.

The main achievements of the procedure are:

1) Potential targets for indicaxanthin have been identified. They include Inositol Trisphosphate 3-Kinase (ITP3K), Glutamate carboxypeptidase II (GCPII), Leukotriene-A4 hydrolase (LTA4H), Phosphoserine phosphatase (HPSP), Phosphodiesterase 4D (PDE4D), AMPA receptor (GluA3 and GluA2 sub-units) and Kainate receptor (GluK1 isoform). These targets are implicated in neuromodulation, and inflammatory regulation, normally expressed mostly in the CNS, and expressed (or overexpressed) in cancer tissues (i.e. breast, thyroid, and prostate cancer cells).
2) Qualitative and quantitative information about dynamic interactions of indicaxanthin at the binding site of target proteins have been highlighted.

The results of the docking procedure are summarized in Table 2, in which also calculated free energies of bindings are listed. Altogether these findings confirm the experimental data that consider indicaxanthin as a molecule of biological interest as already highlighted in several by *in vivo* and *in vitro* test (see Table 1).

Therefore, indicaxanthin may be further tested as a potential lead molecule for the prevention or the treatment of neurological disease and related cancer pathologies.

## 3. Conclusions and perspectives

In this review we reported an excursus about indicaxanthin, a natural compound obtained mainly from *Opuntia ficus-indica* (prickly pear). Despite the fact that indicaxanthin and related compounds are usually extracted from natural sources, several synthetic methodologies have been reported in literature. But to date, extraction and purification from vegetal matrices remain the gold standard to obtain indicaxanthin in high yield. From a pharmacological and biochemical perspective, the above-reported effects and mechanisms of action exerted by indicaxanthin in a number of experimental systems fall within the remit of the intense research on the health-promoting power of phytochemicals. Lack of toxicity, high bioavailability, redox-modulating potential, BBB permeability, together with the ability to exert pharmacological effects by modulating several targets in complex pathological conditions are the hallmarks of indicaxanthin pharmacological potential. Along these lines, we propose indicaxanthin as a novel therapeutical agent to be further explored in more complex studies in combo therapy, i.e. with other therapeutical agents targeting different check points of the disease progression. Last but not least, we described computational efforts to determine the biological targets and pKa values. These latter could explain the antioxidant power of indicaxanthin. We are aware that further use of computer-assisted methodologies will help the mechanistic interpretation of the biological activity of indicaxanthin.

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