Natural Coumarins as a Novel Class of Neuroprotective Agents

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Abstract: Neurodegenerative disorders are a heterogeneous group of diseases and are among the most invaliding syndromes for humans. The aim of this manuscript is to review what has been reported so far in the literature about coumarins as a novel class of pharmacologically active compounds in chemoprevention and/or therapy of these diseases.

Key Words: Chemoprevention, coumarins, natural compounds, neurodegenerative disorders, neuroprotective activity.

INTRODUCTION

Neurodegenerative diseases represent a widely differentiated group of syndromes of the nervous system, including the brain, spinal cord and peripheral nerves, arising from several causes, many of which are still unknown [1]. Several of these diseases are hereditary, some are consequences of toxic or metabolic processes others result from microbial infections and finally for others causes remain unknown. Due to the incidence, morbidity and mortality of the neurodegenerative syndromes, they represent a huge problem from a medical, social and financial point of view for human society. Pathologically, neurodegeneration result from abnormalities in the function of specific regions of the central and peripheral nervous systems and/or specific populations of neurons. Depending on which cluster of neurons begin to undergo pathological changes this will lead to the clinical phenotype of that particular illness. Recent studies have allowed the identification of a certain number of genes responsible for several neurodegenerative diseases. Moreover, during the last few years, valuable animal models have been developed to study factors and mechanism underlying the etiology and pathogenesis of these syndromes. Currently used therapeutic approaches are able to treat the symptoms for a limited period of time but, as the underlying progression of the disease advances, these treatments become ineffective. As stated before, neurodegenerative syndromes have multifactorial causes, so it's reasonable that different therapeutic approaches have been proposed depending on which biological target has to be triggered. Drugs more commonly used are those able to restore the imbalance in neurotransmitter function that is a peculiar feature of the most part neurodegenerative symdromes. Some examples are the use of L-DOPA in combination with DOPA-decarboxylase inhibitors in the treatment of Parkinson's disease and the use of cholinomimetics and acetylcholinesterase inhibitors in the therapy of Alzheimer's disease. Other drugs in the therapeutic management of neurodegenerative disorders include inhibitors of key enzymes like COX-2 [2], MAO-B [3], *i*NOS [4]and caspase [5], heavy metal chelators [6], radical and reactive oxygen species scavengers [7,8], statins [9] and finally the most recent approaches are genetic [10] and stem-cell [11] based therapies. In recent years natural products have been re-discovered as a valuable source of novel agents exerting positive effects in the treatment of syndromes like cancer and microbial infections, against which synthetic drugs failed due to progressively increasing pharmacological resistance. In particular several natural compounds have been seen in the last decade to efficiently interfere at different levels with the development and progress of many neurodegenerative disorders. These include ferulic acid **1** [12], oleamide **2** [13], flavonoids like naringenin **3** [14], baicalein **4** and baicalin **5** [15], catechins from Green tea [16], tocopherols [17], curcumin **6** [18] and folate **7** [19] (Fig. **1**).

Coumarins represent a large class of phenolic compounds widely distributed in plants, mainly those belonging to families of Rutaceae and Apiaceae, but also in fungi and bacteria.

To date more than 1300 compounds belonging to this group of secondary metabolites have been identified [20]. Coumarins have recently became of great interest because of their pharmacological activities. In fact they have been reported to exert several and different biological activities [21]. They have been used to treat diverse syndromes such as cancer, burns, cardiovascular and rheumatic diseases [20]. The coumarin moiety has been also shown to be a fundamental structural requirement for the unique anti-oedema and anti-inflammatory activities exerted by these natural products. Several semi-synthetic coumarin derivatives are not only recognized as effective inhibitors of enzymes along the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism [22-24], but also of neutrophile dependent superoxide anion generation [25]. Several natural or synthetic polyhydroxylated and polymethoxylated coumarins were found to efficiently inhibit lipid peroxidation, to behave as scavenger of hydroxyl radicals and superoxide anions [26] and to influence processes involving free radical-mediated injury with the same degree of activity as other plant phenolics like flavonoids [27]. Although well characterized for many biological activities, only in the last decade have natural coumarins been seen as potential pharmacological agents

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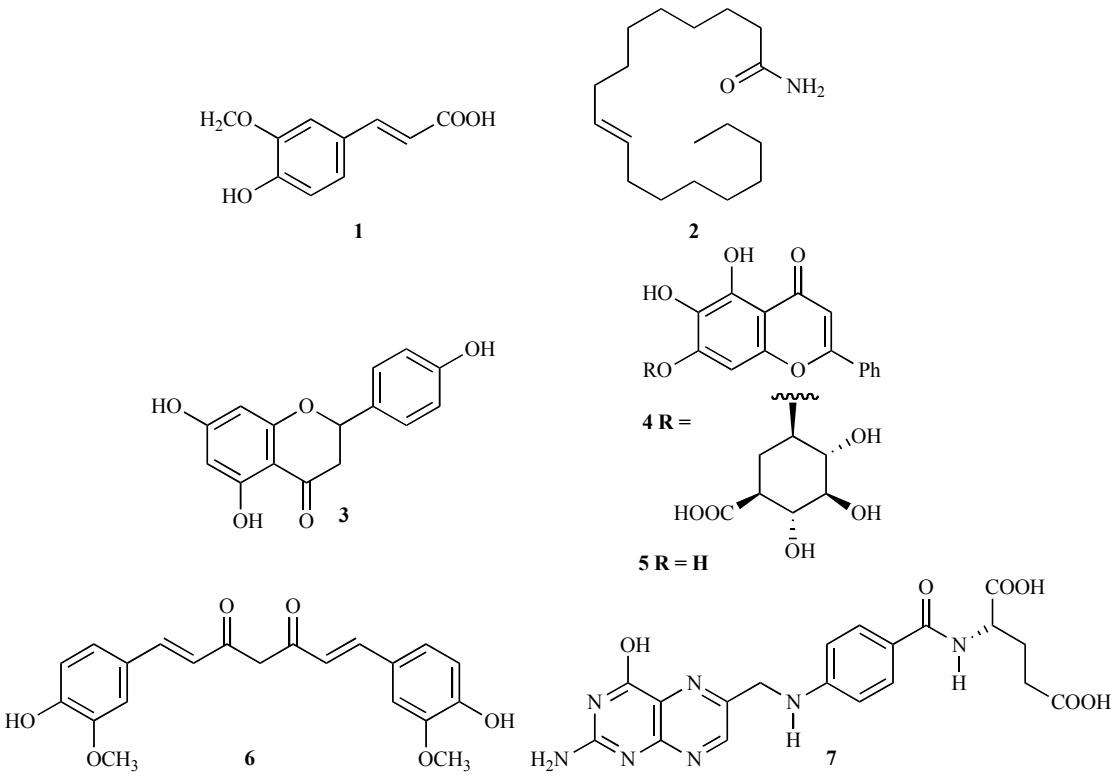

Fig. (1). Structure of reported neuroprotective natural products.

acting on neurological syndromes. Coumarins that have been reported to act in this way are fraxetin **8**, the main coumarin extracted from *Fraxinus* spp., sunflower oil and some edible vegetables [28-29], imperatorin **9**, isopentenyloxycoumarin **10**, both most commonly found in plants belonging to the genus *Citrus* and *Angelica* [30] and finally a group of coumarins extracted from roots of *Angelica gigas* Nakai [31] (Fig. **2**). So the aim of this manuscript is to review what has been reported so far in the literature about the above cited coumarins as a novel class of pharmacologically active agents in chemoprevention and/or therapy of neurodegenerative diseases.

FRAXETIN

Benedí and coworkers studied exhaustively the properties of fraxetin 8 as neuroprotective agent. In the first of a series of papers they studied the modifications induced by this coumarin on endogenous antioxidant defense system in the brain [32]. In particular, they evaluated oxidative stress indices including superoxide dismutase (SOD), catalase (CAT), total and selenium-dependent glutathione peroxidases (GPx) and glutathione reductase (GR) activities, glutathione disulphide (GSSG) / reduced glutathione (GSH) ratio and thiobarbituric acid-reactive substances (TBARs) concentration in brain supernatants of C57BL/6J male 12-month-old mice treated with fraxetin for 30 days.

Data from this experiment revealed that SOD and GPx activities increased while a substantial decrease was recorded for GSSG / GSH ratio and for the rate of accumulation of TBARs. The authors hypothesized that fraxetin could act against free radical mediated events leading to progression of degenerative diseases of ageing. Some years later, the same research group focused its attention on the role of fraxetin in the rotenone-induced apoptosis in human neuroblastoma dopaminergic SHSY5Y cells, employed as a neuronal model of Parkinson's disease. They evaluated the activity of fraxetin compared with two known antioxidant agents, myricetin, a

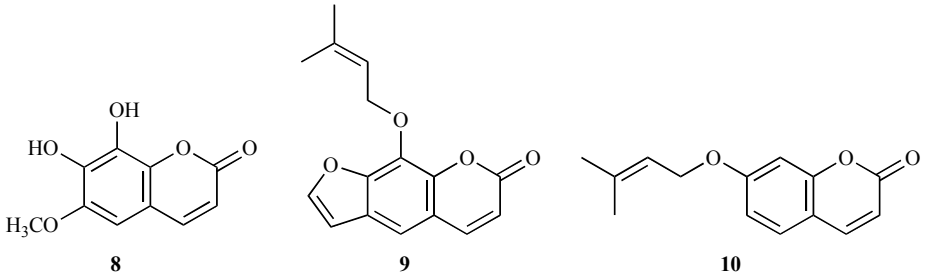

Fig. (2). Structure of the reported neuroprotective coumarins.

widespread flavonoid and *N*-acetylcysteine [33]. In particular this study determined cell viability by the MTT method, cytotoxicity by measuring lactate dehydrogenase (LDH and parameters related to apoptosis such as caspase 3 activity, poly(ADP-ribose) polymerase (PARP) cleavage and the levels of reactive oxygen species (ROS). These factors are known to be involved in the pathogenesis of Parkinson's disease. In these experiments cells were pre-treated with myricetin 50 μM, fraxetin 100 μM and *N*-acetylcysteine 100 μM, 30 min. before incubation for a period of 16 h with rotenone at a concentration of 5 μM. Rotenone was found to induce apoptosis in SHSY5Y cells in time- and dose-dependent manner and induced LDH release. Fraxetin alone exhibited a significant level of neuronal death, although only at high concentration (500 μM), while myricetin decreased cell viability even at 100 μM. Fraxetin and *N*-acetylcysteine both at the dosage indicated above protected cells against neurotoxicity induced by rotenone, while myricetin had no significant activity. Also fraxetin induced a level of LDH release equal to that evoked by *N*-acetylcysteine, while the effect of the flavonoid substance was minor. Treatment of cells with rotenone 5 μM alone led to a significant increase of both caspase-3 activity and cleavage of PARP. SH-SY5Y cells treated with myricetin 50 μM, fraxetin 100 μM and *N*-acetylcysteine 100 μM for 30 min prior to exposure to rotenone 5 μM for 16 h resulted in an appreciable decrease in caspase activity by 21%, 85% and 40% respectively, compared with cells treated only with the cytotoxic agent. While myricetin showed only a partial effect on cleavage of PARP induced by rotenone, fraxetin and *N*-acetylcysteine almost completely inhibited this effect.. Finally, treatment with rotenone induced a rapid and massive production of ROS from 96.4 ± 5.4 (control) to 152.6 ± 23.7 (rotenone alone). Pretreatment with myricetin, fraxetin and N-acetylcysteine not only suppressed the generation of ROS in SH-SY5Y cells but significantly decreased the amount of intracellular peroxide levels to 119.3 ± 9.5 (myricetin [50 μM] + rotenone), 62.3 ± 3.4 (*N*-acetylcysteine [100 μM] + rotenone) and 43.7 ± 16.3 (fraxetin [100 μM] + rotenone). Data from this study showed for the first time that among natural products, also coumarins could lead to beneficial effects concerning neurodegenerative diseases in a better manner than other secondary metabolites like flavonoids, that have been claimed as a "panacea" for oxidative stress-induced syndromes. Benedí and coworkers evaluated the morphological changes induced by rotenone and intracellular ROS production in the same cell line as above by means of confocal microscopy using fluorescent dyes such as dihydroethidium (HE) and 2',7'-dichlorofluorescein diacetate (DCFH-DA), specific probes for detection of production of H_2O_2 and superoxide anion respectively, DNA fragmentation extent as index of apoptosis and oxidative stress parameters such as glutathione redox status and unsaturated lipid peroxidation [34]. Treatment of SHSY5Y cells with rotenone 5 μM for 16 h resulted in widespread cell death and most cells lost neuritis and showed round shape with some lysing or replaced by debris. Pretreatment with fraxetin prevented many of the observed morphological changes. The percentage of death in (cells that were positively stained with propidium iodide) in cells treated with rotenone was 42 ± 3.8 %, while this value decreased to 13 ± 1.2 % in the group treated with the coumarin, an extent comparable to *N*-acetylcysteine (11 ± 1.1 %). Rotenone, 5 μM after 16 h, increased the production of ROS (H_2O_2 and O_2^-). Pretreatment with fraxetin led to a reduction of 75 ± 9.9 % and 93 ± 6.7 % for the production of H_2O_2 and superoxide anion respectively. While rotenone yielded a wide DNA ladder resulting from fragmentation of internucleosomal DNA into specific oligonucleosomal DNA this effect was reduced by fraxetin. Basal level of GSH and GSSG in control cells were 37.45 ± 2.7 and 18.41 ± 1.4 nmol/mg protein, respectively. Treatment with rotenone led to an increase of 56 ± 2.4 % and 35 ± 6.0 % of the control values for GSH and GSSG respectively. In contrast, treatment with fraxetin resulted in an increase of basal level of GSH relative to untreated cells by 72.23 ± 4.7 % and a decrease of GSSG by 42.21 ± 1.2 %. Compared to rotenone-treated cells, GSH levels were higher in cells treated with fraxetin (91.11 ± 2.4 %). Fraxetin was also able to restore the GSH / GSSG ratio that was significantly decreased by rotenone. Finally, in this study the authors observed peroxidation of cell membrane lipids to a large extent (52 ± 2.1 %) in rotenone-treated cells although only after 24 h. Treatment with fraxetin inhibited the process of lipid peroxidation in rotenone-treated cells. The way by which fraxetin could improve the efficacy and potency of endogenous antioxidant defense system in the brain was studied by the same research group in 2005. They evaluated the protective effects of fraxetin on the activities of MnSOD and CuZnSOD, CAT, GR and GPx in rotenone-treated SH-SY5Y cells [35]. Another marker of oxidative stress in several tissues, the expression of heat shock protein (HSP70), able to protect cells from oxidative stimuli, was measured at mRNA and protein levels in the same cell line. Treatment with rotenone 5 μM led to increase in the activity of the mithocondrial form of SOD (MnSOD) (622.9 ± 56 *vs*. 351.2 ± 29 mU/mg protein recorded for untreated cells), but no significative effect on the cytosolic enzyme (CuZnSOD). Pretreatment with fraxetin lowered the activity of MnSOD to 477.4 ± 59 mU/mg protein. This result was confirmed by Western-blot analysis. Catalase activity increased in a dose-dependent manner in cells treated with rotenone. Among the anti-oxidant agents tested, only fraxetin led to a significative decrease in catalase activity (699.4 ± 67 *vs*. 1122.3 ± 99 mU/mg protein recorded for rotenone treated cells), while treatment of cells with fraxetin alone did not cause appreciable changes in the activity of this enzyme compared to untreated cells. Immunoblot analysis showed that the expression of CAT in respect to rotenone treated cells was reduced selectively by fraxetin. On the other hand, the coumarin did not affect the activity and expression of GR and GPx in rotenone-treated SH-SY5Y cells. Finally it was seen that treatment with rotenone led to only a slight increase in the expression of HSP70, as measured by the concentration of its transcripts. In contrast, pretreatment with fraxetin increased substantially the level of expression of this protein with or without rotenone exposure, compared with cells treated with rotenone alone (1508 ± 136 and 447 ± 40 *vs*. 227 ± 20%, respectively). In their last study on this topic, Benedí and coworkers investigated further the protective effect of fraxetin on endogenous reduced glutathione (GSH), intracellular oxygen species (ROS) and apoptotic death in rotenone-mediated cytoxicity [36]. They also determined changes in the levels of mitochondrial Bcl-2, Bax, caspase-3

and 9 activation and cytochrome c. Incubation for 24 h of fraxetin led to a dose-dependent increase of GSH levels. Fraxetin 10 μM gave a 32 % increase, this value becoming approximately double for a concentration of 100 μM. Rotenone 1 μM alone after 24 h exposure led to the loss of more than 50% of intracellular GSH. Using a known depleting agent of GSH, buthionine sulfoximine (BSO) at a concentration of 50 μM after 24 h, the loss of endocellular GSH reached approximately 62%. Moreover depletion of GSH by BSO treatment increased the cells susceptibility to toxicity caused by rotenone to 18.5%. Pre-treatment with fraxetin (100 μM) markedly attenuated BSO-induced GSH depletion in cells treated with rotenone compared to the group treated with BSO. Treatment with BSO for 24 h prior to treatment with rotenone 1 μM for additional 24 h increased the cytotoxicity of the latter up to approximately 71%. In this case however fraxetin exhibited no significative increase of cell viability, leading to the hypothesis that loss of GSH under these condition is not correlated with cytotoxicity. Treatment with rotenone 1 μM for 24 h gave a 5-fold increase in the release of cytochrome c respect to the untreated cell group. Upon treatment with fraxetin the release of cytochrome c was decreased by 18% when cells were pre-treated with a concentration of 10 μM of coumarin and the inhibitory percentage was 47% when fraxetin was increased to 100 μM. Treatment of SH-SY5Y cells with the same concentration of rotenone along the same period led to an increase of both caspase-3 (290%) and caspase-9 activities (193%). SH-SY5Y cells pre-treated with fraxetin (10, 50, and 100 μM) attenuated rotenone-induced caspase-3 activation about by 25, 82, and 93%, respectively. Fraxetin alone did not show a significant effect on the caspase-3 activity and it did not significantly prevent the increase in the activity of caspase-9. The expression of Bax protein increased by 62% in the rotenone-treated cells compared to the control ones. The ratio of Bcl- 2 to Bax was decreased by 52% by rotenone. Fraxetin, which had no effects in control cells, prevented the increase in the level of Bax observed in the rotenone treated group. Fraxetin (50–100 μM) also attenuated the decrease of the ratio of Bcl-2 to Bax in the rotenone-treated cells. It's noteworthy that in all cases the biological activity displayed by fraxetin was superior to that shown by the flavonoid myricetin and comparable or superior to that of the potent antioxidant *N*-acetylcysteine. In summary, Benedí and coworkers found fraxetin as a valuable neuroprotective agent. The authors hypothesized that fraxetin prevents rotenone-induced cytochrome c release and caspase-3 activation by decreasing mitochondrial Bax and increasing Bcl-2/Bax ratio; fraxetin may also act on ROS to inhibit apoptosis by its antioxidant properties, partially due to its catechol-like structure, since it is known that catechol-containing polyphenols show higher scavenging activity for free radicals than other coumarins without a catechol moiety; it may also modulate the activity of key enzymes part of the endogenous antioxidant defense system in the brain and restore the physiological concentrations of GSH. Although the potential use of fraxetin as a neuroprotective agent may be suggested, the cost-effective availability of this natural product will determine its wider use as a chemopreventive agent because currently the only source of fraxetin is through absorption from food plant (e.g. carrot) diet and as a compound contained in medicinal plants.

IMPERATORIN

The first manuscript dealing with interaction of the oxyprenylated furanocoumarin imperatorin **9** with targets in the CNS wasen reported in 2005 by Baek and coworkers [37]. By means of a bioassay guided fractionation of the methanol extracts of roots of *Angelica dahurica* Bentham and Hooker (fam. Apiaceae), they studied the interaction of imperatorin with GABA transaminase (GABA-T), the enzyme responsible for the degradation and so inactivation of GABA to succinic semialdehyde. Studying the catabolism of this neurotransmitter is crucial as it's now well recognized that the development of several neurodegenerative disorders is correlated to a decrease of GABA in the CNS and the binactivation of GABA-T could represent a target for the therapy of these diseases. They found that **9** inhibited in a time- and dose-dependent manner GABA-T and following a second-order rate kinetic with a constant of 2.3 ± 0.3 mM^{-1} min^{-1}. To demonstrate that imperatorin acted as an active-site-directed irreversible inhibitor of GABA-T activity, Baek and coworkers studied also the ability of the natural substrates of this enzyme (GABA and α-ketoglutarate) to protect GABA against inactivation. Pre-incubation of the isolated GABA-T with 10 mM α-ketoglutarate or 20 mM GABA protected the neurotransmitter from deamination by 98% and 74% respectively. Based on this data the authors hypothesized that inactivation of GABA-T by imperatorin could be due to a selective modification of a functional group in the active site rather than coming from a nonspecific reaction mechanism. Also considering the fact that agents able to potentiate GABA neurotransmission in the brain could be considered as potential anti-epileptic drugs, Luszczki and coworkers in 2007 investigated the effect of imperatorin on the anticonvulsant activity of four conventional antiepileptic drugs (carbamazepine, phenobarbital, phenytoin and valproate) in the mouse maximal electroshock seizure model (MES) [38]. Moreover they studied the combinations of imperatorin with conventional antiepileptic drugs in relation to impairment of motor coordination, long-term memory and muscular strength by the use of the chimney test, step-through passive avoidance task and grip-strength test, and measured total brain antiepileptic drug concentrations in order to ascertain whether any observed effects were consequent to a pharmacodynamic and/or a pharmacokinetic interaction. Imperatorin (50 mg/kg administered alone, i.p., 30 min prior to the test) dose-dependently raised the median current strength (CS_{50}) values necessary to produce tonic hindlimb extension in 50% of animals from 6.95 mA to 9.59 mA. Effects of imperatorin on the protective action of the above cited antiepileptic drugs in the mouse maximal electroshock-induced seizure model are reported in Table **1**.

When imperatorin at 30 and 40 mg/kg was co-administered with carbamazepine, it significantly enhanced the anticonvulsant effect of this drug against MES by reducing its ED_{50} value from 10.3 to 6.8 and 6.0 mg/kg (34% and 42%), respectively. In the case of phenobarbital, imperatorin at a dose of 40 mg/kg significantly enhanced the anti-electroshock

Table 1. Effects of Imperatorin 9 on the Protective Action of Common Antiepileptic Drugs in the Mouse Maximal Electroshock-Induced Seizure Model [38]

Treatment (mg/kg)	ED_{50} (mg/kg)	N	S.E.M.
Carbamazepine + vehicle	10.3 (8.7 – 12.2)	16	0.888
Carbamazepine + imperatorin (20)	7.8 (6.3 – 9.7)	24	0.847
Carbamazepine + imperatorin (30)	6.8 (5.4 – 8.7)[a]	16	0.850
Carbamazepine + imperatorin (40)	6.0 (4.8 – 7.5)[b]	24	0.679
Phenobarbital + vehicle	19.6 (15.7 – 24.4)	8	2.182
Phenobarbital + imperatorin (20)	18.7 (15.4 – 22.8)	16	1.880
Phenobarbital + imperatorin (30)	17.7 (15.8 – 20.0)	24	1.075
Phenobarbital + imperatorin (40)	12.2 (9.8 – 15.1)	24	1.336
Phenytoin + vehicle	12.8 (11.1 – 14.8)	16	0.943
Phenytoin + imperatorin (20)	10.1 (8.4 – 12.1)	24	0.945
Phenytoin + imperatorin (30)	9.3 (8.0 – 10.8)	16	0.730
Phenytoin + imperatorin (40)	8.5 (6.8 – 10.6)[a]	16	0.955
Valproate + vehicle	247.9 (224.7 – 273.5)	24	12.403
Valproate + imperatorin (20)	221.1 (202.1 – 241.9)	24	10.127
Valproate + imperatorin (30)	219.4 (195.4 – 246.3)	24	12.961
Valproate + imperatorin (40)	213.4 (194.3 – 234.4)	24	10.201

ED_{50} = median effective doses required to protect 50% of animals tested against maximal electroshock-induced seizures. All drugs were administered systematically (i.p.): phenytoin at 120 min., Phenobarbital at 60 min., carbamazepine, valproate and imperatorin at 30 min. prior electroshock-induced seizures. N = total number of animals; SE = standard error for ED_{50} values.

[a] $p < 0.05$, $p < 0.01$ respect to control group.

action of phenobarbital by decreasing its ED_{50} value from 19.6 to 12.2 mg/kg (38%). Imperatorin at 40 mg/kg markedly potentiated the anticonvulsant effects of phenytoin by decreasing its ED_{50} value from 12.8 to 8.5 mg/kg (34%). Imperatorin at doses of 20, 30 and 40 mg/kg did not significantly affect the anticonvulsant effect of valproate. Administered in combination with the four above cited drugs at its ED_{50} values, imperatorin did not affect behaviour of treated animals in respect to the control group in motor performance as assessed by the chimney, passive avoidance and grip-strength tests. Effects of imperatorin on total brain concentrations of antiepileptic drug in mice, assayed by fluorescence polarization immunoassay method, are reported in Table **2**.

Imperatorin (30 mg/kg) increased total brain carbamazepine concentrations from 1.260 to 2.328 μg/ml (85%) compared to carbamazepine (at a dose of 6.8 mg/kg) administered alone.

In contrast, total brain concentrations of phenobarbital and phenytoin, were not changed significantly after administration of imperatorin. This kind of selectivity in increase in total brain carbamazepine concentration could be explained by the hypothesis that imperatorin might increase the penetration of carbamazepine into the brain by modifying the blood brain barrier permeability. Alternatively it may be supposed that the selective increase in carbamazepine content in the brain tissues resulted from imperatorin-induced inhibition of multidrug resistance proteins or P-glycoproteins, whose normal physiological activity is related to the removal of drugs from this district. It seemed also that the potentiation by imperatorin of effects of carbamazepine versus its effect on phenobarbital and phenytoin derives from different basis, being possible to hypothesize a pharmacokinetic rea-

Table 2. Effects of Imperatorin 9 on Total Brain Concentrations of Antiepileptic Drug in Mice [38]

Treatment (mg/kg)	Brain Concentration (μg/ml)
Carbamazepine (6.8) + vehicle	1.260 ± 0.218
Carbamazepine (6.8) + imperatorin (30)	2.328 ± 0.238[a]
Phenobarbital (12.2) + vehicle	6.101 ± 0.401
Phenobarbital (12.2) + imperatorin (40)	6.303 ± 0.328
Phenytoin (8.5) + vehicle	1.213 ± 0.144
Phenytoin (8.5) + imperatorin (40)	1.145 ± 0.114

[a] $p < 0.001$ respect to carbamazepine + vehicle treated group.

son in the case of carbamazepine and a pharmacodynamic one for the other two drugs. The lack of efficacy of imperatorin on the antiseizure action of valproate could be explained by the fact that valproate possesses a number of various mechanisms of action that contribute to its antiseizure activity in both rodents and humans, considering which it could be supposed that imperatorin competed with valproate to inhibit GABA-T and thus, imperatorin did not potentiate the antiseizure effects of valproate against MES in mice. Recently, using the same experimental animal model and conditions, Luszczki and coworkers investigated the influence of imperatorin on the anticonvulsant activity and acute-adverse effect potential of lamotrigine, a second-generation antiepileptic drug [39]. In this study, imperatorin administered i.p. 30 min before the test, at a dose of 50 mg/kg significantly enhanced the anticonvulsant action of lamotrigine in the MES test, reducing its by 60% from 6.11 to 2.47 mg/kg (Table **3**).

However imperatorin did not significantly affect the acute adverse effects of LTG in the chimney test. Imperatorin 50 mg/kg did not significantly affect the total brain concentration of lamotrigine following its administration at 2.5 mg/kg. Like phenytoin and phenobarbital, the potentiation of the effect of lamotrigine by imperatorin seemed to have pharmacodynamic basis.

7-ISOPENTENYLOXYCOUMARIN

In a study devoted to evaluate the neuroprotective effect of selected prenyloxyphenylpropanoids, Epifano and coworkers assessed the role of 7-isopentenyloxycoumarin **10** as an *in vitro* protective agent against NMDA-induced toxicity in mixed cortical cultures containing both neurons and astrocytes [40]. 7-Isopentenyloxycoumarin exerted its effect in a dose dependent manner (Table **4**) reaching the highest value of neuroprotection (49.5 %) at the dose of 100 μM.

It has been seen also that only compounds having a coumarin moiety exerted a neuroprotective effect, while other prenyloxyphenylpropanoids sharing the presence of a carboxylic group (prenyloxycinnamic and prenyloxybenzoic acids) were virtually not active. Moreover a single isoprene unit rather a geranyl one as *O*-side chain attached to the coumarin ring seemed to be more effective to this aim. Concerning the mechanism of action underlying the observed effect of compound **10** that 7-isopentenyloxycoumarin could act as inhibitor of acetylcholinesterase, MAO-B and Ca^{+2} efflux through cell membranes leading so to an overall potentiation of cholinergic transmission in the CNS. Although previous studies have described several natural and semisynthetic coumarins to be effective protective agents against neuronal damage induce by excitotoxic aminoacids or oxidative stress promoting compounds, this represents the first report describing natural linear prenyloxycoumarins as neuroprotective compounds.

Table 4. Dose-Response Curve of 7-Isopentenyloxycoumarin 10 as Neuroprotective Agent Against NMDA-Induced Toxicity in Mixed Cortical Cultures [40]

Dose (μM)	% of NMDA Toxicity[a]
0	99.81 ± 5.98
0.1	88.09 ± 11.72
1.0	63.22 ± 14.91
10	55.57 ± 4.84
100	50.50 ± 3.68

[a]$p < 0.05$ at Student's *t* test; data expressed as mean ± SEM.

COUMARINS FROM *ANGELICA GIGAS* NAKAI

Plants belonging to the genus *Angelica* are a good source of several kind of coumarins. Although known for long time, the first study describing the isolation and neuroprotective effects of coumarins from *A. gigas* Nakai appeared only in 2005. By means of a bioassay-guided fractionation, Kim and coworkers isolated from the roots four new decursin and decursinol derivatives, namely 4"-hydroxytigloyldecursinol **11**, 4"-hydroxydecursin **12**, (2"*S*,3"*S*)-epoxyangeloyldecursinol **13** and (2"*R*,3"*R*)-epoxyangeloyldecursinol **14** exhibiting this kind of biological effect together with the already known decursin **15** and decursinol **16** (Fig. **3**) [41]. In particular they found that these natural compounds exhibited significant protective activity against glutamate-induced neurotoxicity when added to primary cultures of rat cortical cells at concentrations ranging from 0.1 to 10 μM.

The protective effects against glutamate-induced neurotoxicity were measured by determining the LDH activity released in the medium. Results are reported in Table **5**.

Among the compounds tested, (2″*S*,3″*S*)-epoxyangeloyldecursinol **13**, (2″*R*,3″*R*)-epoxyangeloyldecursinol **14**, decursin **15** and decursinol **16** exhibited similar efficacies, showing a relative protection (60 - 70%) in neuroprotective activity, although significant differences in their potencies

Table 3. Effects of Imperatorin 9 on the Anticonvulsant Activity Profile of Lamotrigine in the MES-Induced Seizures in Mice [39]

Treatment (mg/kg)	ED_{50} (mg/kg)	N	SE
Lamotrigine + vehicle	6.11 (4.52 – 8.25)	24	0.938
Lamotrigine + imperatorin (20)	5.77 (4.28 – 7.77)	8	0.876
Lamotrigine + imperatorin (30)	4.28 (2.79 – 6.58)	16	0.939
Lamotrigine + imperatorin (40)	2.47 (1.22 – 4.99)[a]	24	0.886

Drugs were adiministered i.p. as follows: lamotrigine at 60 min. and imperatorin at 30 min. prior to the MES test;. [a]$p < 0.05$.

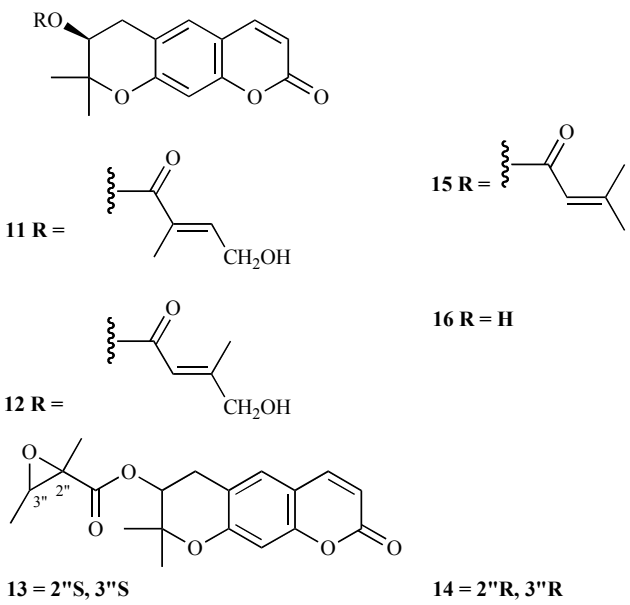

Fig. (3). Structure of neuroprotective coumarins isolated from roots of *Angelica gigas* Nakai.

were observed. In particular, (2″*S*,3″*S*)-epoxyangeloyl-decursinol exhibited the highest neuroprotective effect, 70.0% at 0.1 μM, showing also a significant protection of 60.2 ± 5.3% at 0.01 μM. Moreover it's noteworthy that (2″*S*,3″*S*)-epoxyangeloyldecursinol was revealed to be equally effective as a known noncompetitive antagonist of NMDA receptor, MK-801, and more potent than it. Both 4″-hydroxytigloyl-decursinol and 4″-hydroxydecursin showed slight neuroprotective effects, suggesting that the hydroxylated five-carbon units at C-3′ of decursinol reduced the neuroprotective activity of the decursinol moiety. Kang and Kim isolated other coumarins from *n*-butanol soluble fraction of the extract and found that three of these, the dihydrofuranocoumarins marmesinin **17**, nodakenin **18** and columbianetin-*O*-β-D-glucopyranoside **19** (Fig. **4**) exerted significant neuroprotective activities in the same experimental model as described above, exhibiting cell viabilities of 15.4 – 50.4 % at concentrations ranging from 0.1 to 10 μM [41].

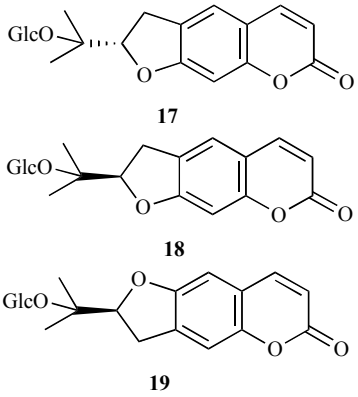

Fig. (4). Structure of neuroprotective coumarins isolated from *n*-butanol extract of roots of Angelica *gigas* Nakai.

To explore the structure-activity relationships of isolated coumarins from *A. gigas*, Kim and coworkers tested twenty-five compounds using their *in vitro* experimental model. Results are reported in Table **6**.

The authors classified the neuroprotective activities of most of the twenty-five coumarins in the following order:

Table 5. Neuroprotective Effects of Compounds 11-16 on Primary Cultures of Rat Cortical Cells Injured by Glu (100 μM) Treatment for 24 h [41]

		Cell Viability (%)	
Compound	**0.1 μM**	**1 μM**	**10 μM**
Control		100.0	
Glu-treated		0.0	
11	33.2 ± 3.5*	19.9 ± 2.0	1.2 ± 4.0
12	38.4 ± 4.0*	34.1 ± 3.5*	35.1 ± 4.5*
13	70.0 ± 6.0***	52.5 ± 4.4**	49.0 ± 3.0**
14	47.5 ± 4.0**	61.1 ± 5.0**	56.7 ± 2.8**
15	39.0 ± 2.6*	0.0 ± 3.3**	65.1 ± 1.6***
16	33.4 ± 3.6*	69.6 ± 6.6***5	40.7 ± 6.9*
MK-801	54.0 ± 5.0**	65.0 ± 5.0***	70.0 ± 6.2***
APV	10.0 ± 2.5	25.0 ± 3.0	39.0 ± 4.0*
CNQX	29.0 ± 3.5*	40.5 ± 3.7*	50.5 ± 4.5**

MK-801: dizocilpine maleate (non competitive NMDA receptor antagonist); APV: DL-2-amino-5-phosphonovaleric acid, (competitive NMDA receptor antagonist); CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione (non-NMDA receptor antagonist); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 6. Neuroprotective Effects of Selected Coumarins from *A. gigas* Against Glutamate-Induced Toxicity in Primary Cultured Rat Cortical Cells [31]

Compound	Cell Viability (%)		
	0.1 μM	1 μM	10 μM
Control		100.0	
Glu-treated		0.0	
Marmesinin **17**	45.9 ± 5.5**	50.4 ± 2.5**	40.4 ± 6.0*
Nodakenin **18**	15.4 ± 0.5	34.5 ± 5.5*	49.4 ± 6.1**
Columbianetin-β-D-glucopiranoside **19**	43.6 ± 3.9*	48.4 ± 2.9**	40.7 ± 3.2*
(S)-Peucedanol-7-*O*-β-D-glucopiranoside **20**	31.4 ± 4.0*	50.3 ± 5.0**	32.4 ± 6.7
(S)-Peucedanol-3'-*O*-β-D-glucopiranoside **21**	31.3 ± 6.0	32.9 ± 5.0	39.5 ± 4.8*
Skimmin **22**	26.0 ± 5.5	16.9 ± 3.2	12.0 ± 3.9
Apiosylskimmin **23**	22.2 ± 4.7	24.1 ± 3.1	12.0 ± 3.0
Isoapiosylskimmin **24**	35.9 ± 4.0*	34.1 ± 6.0	20.0 ± 4.0
Magnolioside **25**	34.7 ± 3.0	12.2 ± 5.5	10.2 ± 3.0
Umbelliferone **26**	35.0 ± 3.9*	36.6 ± 3.0*	9.5 ± 1.5
Demethylsuberosine **27**	36.0 ± 4.5*	44.0 ± 1.5**	39.5 ± 4.0*
7-Hydroxy-6-(2-(R)-hydroxy-3-isopentenyl)coumarin **28**	45.5 ± 3.0**	29.9 ± 1.2*	17.0 ± 3.5
Peucedanone **29**	28.6 ± 2.5	40.7 ± 4.0*	35.0 ± 4.1*
7-Methoxy-5-prenyloxycoumarin **30**	20.0 ± 3.5	18.0 ± 2.0	15.5 ± 3.0
Xanthotoxin **31**	40.0 ± 4.0*	42.0 ± 5.0*	35.0 ± 6.0
Isoimperatorin **32**	54.7 ± 6.8**	51.7 ± 2.9**	27.1 ± 4.0
Marmesin **33**	50.1 ± 4.1**	53.0 ± 5.7**	57.2 ± 6.7***
Nodakenetin **34**	42.8 ± 5.0*	54.0 ± 4.5**	47.0 ± 3.8**
Xanthyletin **35**	12.0 ± 3.0	22.1 ± 4.9	19.0 ± 3.0
Decursinol **16**	33.4 ± 3.6*	69.6 ± 6.6***	40.7 ± 6.9**
Decursin **17**	39.0 ± 2.6*	50.0 ± 3.3**	65.1 ± 1.6***
4"-Hydroxytigloyldecursinol **11**	33.2 ± 3.5*	19.9 ± 2.0	1.2 ± 4.0
4"-Hydroxydecursin **12**	38.4 ± 4.0*	34.1 ± 3.5*	35.1 ± 4.5*
(2"S-3"S)-Epoxyangeloyloxydecursinol **13**	70.0 ± 6.0***	52.5 ± 4.4**	49.0 ± 3.0**
(2"S-3"S)-Epoxyangeloyloxydecursinol **14**	47.5 ± 4.0**	61.1 ± 5.0***	56.7 ± 2.8**
MK-801	53.0 ± 5.0**	65.0 ± 5.0***	71.0 ± 5.2***
APV	11.0 ± 2.5	25.0 ± 3.0	41.0 ± 4.0*
CNQX	29.0 ± 3.5*	42.0 ± 3.7*	52.5 ± 5.0**

* $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$.

dihydropyranocoumarins > dihydrofurano and furanocoumarins > 7-hydroxycoumarins with or without a prenyl group. It's noteworthy that the neuroprotective activities of the two epoxyangeloyldecursinols **13** and **14** and decursinol **16** were comparable to that of MK-801, the most effective among the three reference drugs used. The activities of **13** and **14** appeared to be superior than those without an epoxide ring **11** and **12**, showing that the epoxidation of the hydroxylated prenyl unit may enhance the neuroprotective activity. With the only exception of xanthyletin **35**, pyranocoumarins **11-16** exerted a valuable neuroprotective activity, suggesting the dihydropyran moiety of decursinol may play an important role in this kind of effect.

In addition, five dihydrofuranocoumarins **17-19**, **33** and **34** and a furanocoumarin, isoimperatorin **32** also significantly attenuated the glutamate-induced toxicity, leading to cell viabilities of 50-60% at concentrations ranging from 0.1 to 10 μM.

This reinforces the hypothesis that the cyclization of the prenyl chain yielding dihydropyran or dihydrofuran rings is crucial for the observed activity. The aglycones of compounds **17** and **18**, marmesin **33** and nodakenetin **34**, showed a greater effect compared to the corresponding glycosides. In addition, umbelliferone **26** exhibited a significant neuroprotective activity while, at the same concentration range of 0.1 and 1 μM, its glycosides; skimmin **22** and apiosylskimmin **23**, showed no appreciable effect. These results suggest that the enhanced hydrophilicity due to addition of a sugar moiety may reduce the neuroprotective activity of these aglycones. The addition of a lipophilic moiety to the coumarin ring may facilitate the penetration of these derivatives through cell membranes.

CONCLUSIONS AND FUTURE PERSPERCTIVES

In this review we outlined what has been reported so far in the literature about natural coumarins seen as a novel class of neuroprotective agents. Although few data have been cited, these secondary metabolites are able to display multiple mechanism of actions, depending on their peculiar structure, as anti-oxidants and radical scavengers, inhibitors of key enzymes in the CNS like acetylcholinesterase and MAO A and B, inhibitors of COX-2, inhibitors of GABA-T, agents acting at different levels in the biosynthesis and metabolism of lipids and many others.

Each of the above cited targets have been claimed as factors playing a crucial role in the pathogenesis of neurodegenerative disorders. For this reason natural coumarins could be effectively seen as valuable neuroprotective agents able to act on multiple biological targets. Another point that is noteworthy is that many of these natural coumarins are contained in edible vegetables and fruits commonly consumed as food or used in ethnomedical folk traditions as medicinal plants in many parts of the world. So elucidating the effects and mechanism of action of these compounds could contribute to evaluate better their role as chemopreventive agents of neurodegenerative syndromes. It has already been seen that the combination of dietary anti-oxidants with ROS scavengers like coumarins, could provide better therapeutic advantages for the management of Parkinson's and possibly other neurodegenerative disorders, than those achievable by the single agent [42]. Data reported herein could be helpful in considering natural coumarins as novel lead compounds in the field of treatment of neurodegenerative syndromes and to enhance efforts devoted to further isolate and characterize from a phytochemical and pharmacological point of view in the near future several more coumarins from natural sources and to develop novel routes to obtain semi-synthetic derivatives exerting more potent neuroprotective activity. Moreover, encouraged by data reported herein, further studies concerning antioxidant and antiepileptic effects of other natural coumarins could be performed so that a detailed structure activity relationship could be made also for this kind of activities.

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