The Spice Sage and Its Active Ingredient Rosmarinic Acid Protect PC12 Cells from Amyloid-β Peptide-Induced Neurotoxicity

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Received December 1, 2005; accepted February 16, 2006

ABSTRACT

Traditional use and clinical reports suggest that the culinary herb sage (Salvia officinalis) may be effective for patients with mild to moderate Alzheimer’s disease (AD). In this study, we evaluated the effect of a standardized extract from the leaves of S. officinalis (SOE) and its active ingredient rosmarinic acid on Alzheimer amyloid-β peptide (Aβ)-induced toxicity in cultured rat pheochromocytoma (PC12) cells. Incubation of PC12 cells with Aβ (fragment 1–42) for 24 h caused cell death, and this effect was reduced by SOE and its active ingredient, rosmarinic acid. Rosmarinic acid reduced a number of events induced by Aβ. These include reactive oxygen species formation, lipid peroxidation, DNA fragmentation, caspase-3 activation, and tau protein hyperphosphorylation. Moreover, rosmarinic acid inhibited phosphorylated p38 mitogen-activated protein kinase activation. These data show the neuroprotective effect of sage against Aβ-induced toxicity, which could validate the traditional use of this spice in the treatment of AD. Rosmarinic acid could contribute, at least in part, for sage-induced neuroprotective effect.

Alzheimer’s disease (AD) is the most common form of dementia and is characterized by progressive impairment in cognitive function and behavior (Mattson, 2000; Nordberg, 2004). In elderly people, AD represents the most frequently occurring form of dementia, especially if considered alongside concomitant cerebrovascular disease (Bullock, 2004). The pathological features of AD include neuritic plaques composed of amyloid-β peptide (Aβ) cores, neurofibrillary tangles (NFT) of hyperphosphorylated tau protein, and neurotransmitter deficit (Nordberg, 2004). Although amyloid plaques and NFT are the main histopathological hallmarks of AD, a primary role for Aβ deposits has been inferred from the association between familiar AD and mutations in the genes that encode the amyloid precursor protein, preselinin-1 and preselinin-2 (Hardy and Selkoe, 2002). Applied to neurons in culture, Aβ induces neuronal death and hyperphosphorylation of tau protein; the latter is the main event responsible for NFT formation in AD brains (Caricasole et al., 2003). However, the precise molecular events that determine the death of neurons challenged with Aβ are unclear. Oxidative stress, apoptosis, cell cycle re-entry, G protein activation, modification of calcium homeostasis, the Wnt pathway disruption, activation of mitogen-activated protein (MAP) kinase cascade, and formation of soluble toxic factors, such as Aβ fragments, are all suggested to play causative roles in Aβ neurotoxicity (Kubo et al., 2002). Notably, oxidative stress leads to activation of stress-activated protein kinases (Puig et al., 2004). One stress-activated protein kinase in particular, p38 MAP kinase, appears to be crucial in AD because it promotes tau protein hyperphosphorylation (Puig et al., 2004).

Based on a retrospective review of the historical role of a number of European herbs in the improvement of cognition and memory, it was shown that the culinary herb sage (Salvia officinalis L, Fam. Labiatae) might potentially provide natural treatment for AD (Perry et al., 1999). A randomized double-blind clinical study has recently shown that an ethanolic extract from S. officinalis is effective in the man-
of mild to moderate AD (Akhondzadeh et al., 2003b).

The clinical relevance of these findings was emphasized by the observation that patients did not experience any adverse effect while taking sage. Pharmacological activities of sage relevant (Howes et al., 2003) to AD include antioxidant activity (Hohmann et al., 1999), anti-inflammatory effects (Baricveic et al., 2001), and cholinesterase inhibition (Perry et al., 1996). However, the precise mode of sage-protective action remains elusive.

To elucidate the pharmacological basis for the use of sage in AD, in this study, we evaluated the effect of a standardized extract from the leaves of *S. officinalis* (SOE) (and its main active ingredient rosmarinic acid) on 1) cell viability, 2) oxidative stress and phosphorylated p38 (p-p38) MAP kinase activation, 3) tau protein phosphorylation, and 4) neuronal apoptosis in cultured rat pheochromocytoma (PC12) cells exposed to Aβ.

Materials and Methods

All of the materials for cell culture were purchased from BioWhittaker (Caravaggio, Italy). Fetal calf serum and horse serum were from Hyclone (Logan, UT). Human β-amylloid peptide (fragment 1–42, Aβ42) and rosmarinic acid were from Tocris Cookson (Bristol, UK). Antibody anti-tau protein recognizing both isoforms of tau and antibody anti-p38 MAP kinase was from BD Biosciences (Milan, Italy); and anti-caspase-3 antibody was from Chemicon (Temecula, CA). Anti-mouse and anti-rabbit IgG were from Dako (Glostrup, Denmark). A hydroalcoholic dry extract from *S. officinalis* aerial parts (SOE) standardized to contain 9.9% rosmarinic acid was a gift from Aboca (S. Sepolcro, Italy). 4-Benzy1-2-methyl-1,2,4-thiadiazol-3,5-dione (TDZD-8) (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide; the final dimethyl sulfoxide concentration in the assay wells was less than 0.01%. This concentration was found to have no effect on the responses under study.

Nerve growth factor (NGF) and all of the other reagents used, unless otherwise stated, were from Sigma (Milan, Italy). Before their use, all of the drugs were dissolved in pyrogen-free distilled water.

Cell Culture. PC12 cells (American Tissue Culture Catalog no. CRL1721) were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 15% horse serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2/95% air. Confluent cells were detached, counted, and seeded into Petri dishes (10-cm diameter) at a density of 1 × 106 cells/ml and allowed to adhere 24 h at 37°C. For neuronal differentiation, the cells were plated in 96-well culture plates at the density of 5 × 104 cells/well and allowed to adhere at 37°C for 2 h. Thereafter, the medium was replaced with fresh medium, and the cells were incubated with Aβ42 (1 μg/ml) in the presence or absence of SOE (0.01–100 μg/ml) or rosmarinic acid (10⁻⁶–10⁻⁴ M = 0.0036–36 μg/ml). After 24 h, 25 μl of MTT (5 mg/ml in Dulbecco’s modified Eagle’s medium) was added to the cells and incubated for an additional 3 h at 37°C. After this time, the cells were lysed, and the dark blue crystals solubilized with 125 μl of a solution containing 50% (v/v) N,N-dimethylformamide and 20% (w/v) SDS, with an adjusted pH of 4. The optical density of each well was measured with a microplate spectrophotometer (Multispec MCC/340; Titrertek, Huntsville, AL) equipped with a 620-nm filter. The cell viability in response to the treatment with Aβ peptide in the presence or absence of SOE (or rosmarinic acid) was calculated as percentage of cell viability = (optical density treated/optical density control) × 100.

Measurement of ROS. The formation of ROS was evaluated by means of the probe 2’,7’-dichlorofluorescein diacetate (DCF) according to the method described previously (Esposito et al., 2006). In brief, PC12 cells were seeded at a density of 5 × 10⁴ cells/well into 96-well plate and allowed to grow for 48 h. 2’,7’-DCF-diacetate (Sigma) was then added directly into the growth medium at a final concentration of 5 μM for 1 h at 37°C. 2’,7’-DCF-diacetate is a nonfluorescent permeant molecule that passively diffuses into cells where the acetates are cleaved by intracellular esterases to form H₂DCF, thereby trapping it within the cell. In the presence of intracellular ROS, H₂DCF is rapidly oxidized to the highly fluorescent 2’,7’-DCF. Therefore, cells were washed twice with phosphate-buffered saline (PBS) buffer, placed in fresh medium, and treated with Aβ42 (1 mg/ml) with or without rosmarinic acid (10⁻⁷–10⁻⁵ M = 0.0036–3.6 μg/ml) for 24 h. After treatment, cells were washed twice with PBS buffer, and plates were placed in a fluorescent microplate reader (LS 55 luminescence spectrometer; PerkinElmer, Boston, MA). Fluorescence was monitored using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Results were expressed as relative fluorescence units (RFU).

Lipid Peroxidation Assay. Malondialdehyde (MDA), the most abundant lipid peroxidation product from PC12 cells, was measured using the thioarbituric acid colorimetric assay. In brief, 24 h after the treatment of the cells with Aβ42 (1 μg/ml) in the presence or absence of rosmarinic acid (10⁻⁶–10⁻⁵ M), the cells were washed three times with 1× PBS, and then they were scraped in 1× PBS containing 0.5 mM EDTA and 1.13 mM butyl-hydroxytoluene. The cell lysis was performed by means of six cycles of freezing and thawing. One milliliter of 10% (w/v) trichloroacetic acid was added directly into the cell lysate to stop the lipid peroxidation. Therefore, cells were harvested and proteins were mixed with gel-loading buffer (50 mM Tris, 10 mM SDS, 0.5 mM dithiothreitol) and incubated in ice for 15 min. The cells were lysed by rapid passage through a syringe needle five or six times, and the cytoplasmatic fraction was then obtained by centrifugation at 13,000g for 1 min.

Western Blot Analysis. Immunoblot analysis was performed on cytosolic fraction of PC12 cells treated as described above, according to previous published work (Esposito et al., 2006). Cytosolic fraction proteins were mixed with gel-loading buffer (50 mM 10% Tris, 10% SDS, glycerol 2-mercaptoethanol/2 mg of bromphenol/ml) at a ratio of 1:4.
Results were expressed as the mean ± S.E.M. of n experiments. Statistical analysis was determined with analysis of variance, and multiple comparisons were performed by Bonferroni test, with P < 0.05 considered significant.

**Results**

**Cell Viability.** The viability of cells in culture was measured by the reduction of MTT (yellow solution) to formazan salt (dark blue crystals). A 24-h incubation of PC12 cells with Aj42 (1 μg/ml) decreased cell viability by 56.5 ± 6.5% (compared with unstimulated cells = 100% viability). Treatment of PC12 cells with either SOE (0.01–100 μg/ml) (Fig. 1A) or rosmarinic acid (10⁻⁸–10⁻⁴ M = 0.0036–36 μg/ml) (Fig. 1B) significantly reduced, in a concentration-dependent fashion, cell death caused by Aβ42. Significant protective effects were achieved starting from the 10 μg/ml (SOE) or 10⁻⁷ M (rosmarinic acid) concentrations. SOE (0.01–100 μg/ml) and rosmarinic acid (10⁻⁸–10⁻⁴ M) did not affect viability in untreated cells (i.e., cells not treated with Aβ42) (data not shown).

**Oxidative Stress.** Oxidative stress was measured both as ROS formation and as quantification of lipid peroxidation of cell membrane. Although ROS formation and MDA were detectable at 1.5 and 6 h, respectively, after Aβ42 maximal effect was observed at 24 h. Therefore, we evaluated the effect of rosmarinic acid at this time point.

Figure 2 shows the effect of rosmarinic acid on ROS formation in PC12 cells treated with Aβ42 (1 μg/ml). ROS accumulation, estimated using a converting reaction of the probe DCFH2 to DCF, was significantly increased after incubation with Aβ42 (1 μg/ml), as indicated by the increase in RFU (95.62 ± 4.09 RFU) compared with unstimulated cells (50.5 ± 2.9 RFU). Incubation of PC12 cells with rosmarinic acid (10⁻⁷–10⁻⁵ M = 0.036–3.6 μg/ml), given 10 min before Aβ42, concentration-dependently reduced the increase in ROS accumulation induced by Aβ42 (Fig. 2A).

Lipid peroxidation was quantified by the thiobarbituric acid colorimetric assay, which measures MDA as the most abundant lipid peroxidation product from cell membranes. An increase in MDA levels indicates that lipid peroxidation occurs. Incubation of PC12 cells with Aβ42 (1 μg/ml) significantly increased MDA levels (100.7 ± 9.3 ng/1 × 10⁶ cells) compared with untreated cells (46.3 ± 4.5 ng/1 × 10⁶ cells). Treatment of PC12 cells with rosmarinic acid (10⁻⁷–10⁻⁵ M = 0.036–3.6 μg/ml), added 10 min before Aβ42, significantly reduced MDA level (Fig. 2B).

**p38 MAP Kinase Activation.** The effect of Aβ42 on p-p38 protein expression, the active form of p38 MAP kinase, was investigated using immunoblot analysis 6 h after Aβ42 (1 μg/ml) addition to the cells. The p-p38 levels were not detectable in unstimulated cells, whereas p-p38 protein expression was markedly increased by Aβ42 (Fig. 3A). In contrast, the levels of p38 did not significantly change among treatments. Hydrogen peroxide (10⁻⁴ M), used as a positive control

![Fig. 1](https://i.imgur.com/3.jpg)  
Fig. 1. Effect of SOE (0.01–100 μg/ml) (A) or rosmarinic acid (10⁻⁸–10⁻⁴ M = 0.0036–36 μg/ml) (B) on Aβ (1 μg/ml)-induced cell death. Cell death was evaluated at 24 h after incubation with Aj42 by the reduction of the tetrazolium salt MTT. SOE (or rosmarinic acid) was added 10 min before Aj42. Results, expressed as the percentage of cell death, are the mean ± S.E.M. of four experiments in triplicate. Untreated cells were assumed to be vital (100% viability). *, P < 0.05, **, P < 0.01, and ***, P < 0.001 versus Aβ42.
untreated cells, i.e., cells not treated with Aβ. Rosmarinic acid was added 10 min before Aβ. Rosmarinic acid was added 10 min before Aβ. Results are the mean ± S.E.M. of three experiments in triplicate. ○○○, P < 0.001 versus control (untreated cells, i.e., cells not treated with Aβ); *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus Aβ.

(Wang et al., 1998), also induced activation of p38. Rosmarinic acid (10⁻⁷–10⁻⁵ M = 0.36–3.6 μg/ml) inhibited Aβ42-induced p38 levels in PC12 cells in a significant and concentration-dependent way (Fig. 3A).

Moreover, compared with p38 MAP kinase activation, GSK-3β activation (i.e., its phosphorylated form, p-GSK-3β) was also evaluated. The p-GSK-3β was weakly expressed in untreated cells, whereas its expression was strongly and significantly increased 24 h after Aβ42 (1 μg/ml) treatment. The expression of p-GSK-3β was almost completely abolished when cells were treated with its highly specific inhibitor TDZD-8 (used as a positive control), whereas rosmarinic acid (10⁻⁶–10⁻⁵ M = 0.36–3.6 μg/ml) did not modify p-GSK-3β expression (Fig. 3B).

**Tau Protein Hyperphosphorylation.** Experiments on tau protein expression, both as nonphosphorylated and hyperphosphorylated tau (pp-tau), are shown in Fig. 4. Western blot analysis showed that nonphosphorylated tau levels were not significantly different during treatments. By contrast, a significant amount of pp-tau was observed in PC12 cells 24 h after exposure to Aβ42 (1 μg/ml). When rosmarinic acid (10⁻⁵ and 10⁻⁴ M = 3.6 and 36 μg/ml) was given 10 min before Aβ42 to PC12 cells, it reduced Aβ42-induced tau hyperphosphorylation.

**Cell Apoptosis.** Cell death was evaluated both as DNA fragmentation and caspase-3 activation in Aβ42-treated PC12 cells.

The morphological characteristics of apoptosis are frequently accompanied by multiple cleavage of DNA into 180- to 200-bp fragments. The oligonucleosomal-sized fragments can be visualized as a characteristic DNA ladder following agarose gel electrophoresis. The formation of mononucleosomes and oligonucleosomes is a well accepted biochemical characteristic of apoptosis. In contrast to untreated cells, DNA extracted from cells treated with Aβ42 (1 μg/ml) showed a ladder pattern, which indicates the formation of mononucleosomes and oligonucleosomes (Fig. 5A). When PC12 cells were treated with rosmarinic acid (10⁻⁶–10⁻⁵ M),
given 10 min before Aβ42, DNA fragmentation was significantly reduced.

We evaluated the appearance of the caspase-3 band in a cellular extract of PC12 cells by Western blot analysis. Analysis of the pro-caspase/total caspase-3 ratio showed that Aβ42 (1 μg/ml) significantly induced apoptosis (as indicated by the decreased ratio) 6 h after treatment, and this decrease was significantly counteracted by rosmarinic acid (10⁻⁶–10⁻⁵ M) (Fig. 5B).

Discussion

The pathophysiology of AD is complex and involves several different biochemical pathways, including defective Aβ protein metabolism, abnormalities of glutamatergic, adrenergic, serotonegic, and dopaminergic transmission, and the potential involvement of inflammatory, oxidative, and hormonal pathways (Evans et al., 2004). Consequently, these pathways are all potential targets for AD treatment and prevention strategies. A range of pharmacological treatments has been tested, including cholinesterase inhibitors, memantine, selegiline, piracetam, vitamin E, anti-inflammatory drugs, and hormone replacement therapy (Evans et al., 2004). However, Cochrane reviews have established efficacy only for memantine and cholinesterase inhibitors (Evans et al., 2004). Because these drugs confer only modest benefits (Doraiswamy, 2002; Evans et al., 2004), additional AD therapies are urgently needed. Research into historical literature has revealed that some activities of sage, particularly its reputation as being good for the memory, may be relevant to AD treatment (Howes et al., 2003). Moreover, recent clinical studies have shown that sage (S. officinalis), as well as the related plant Spanish sage (Salvia lavandulaefolia), is effective in the treatment of mild to moderate AD (Akhondzadeh et al., 2003b; Perry et al., 2003). However, despite these promising clinical observations, the precise mechanism for this herb remains unknown. In the present study, we have shown that in PC12 cells, an extract from the leaves of S. officinalis prevents the neurotoxicity induced by Aβ42, a proteolytic derivative of the large transmembrane amyloid precursor protein, which plays a crucial role in AD (Citron, 2004).
Commercial extracts of sage are commonly standardized to contain discrete amounts (approximately 10%) of rosmarinic acid, an ester of caffeic acid, and 3,4-dihydroxyphenyllactic acid. Rosmarinic acid is also a major ingredient of lemon balm (*Melissa officinalis*), a plant that has shown promising signs of therapeutic activity in patients with AD (Akhondzadeh et al., 2003a). Main activities of rosmarinic acid include antioxidant, anti-inflammatory, antimutagen, antibacterial, and antiviral properties (Petersen and Simmonds, 2003). In the present study, we have shown that rosmarinic acid prevents Aβ42-induced neurototoxicity, as revealed by the MTT assay, suggesting that this compound might be responsible for sag-induced neuroprotection. Significant inhibitory effects were achieved starting from the 10⁻⁷ M concentration, which can be likely achieved following therapeutic administration of rosmarinic acid-containing herbs. Indeed, pharmacokinetic studies in humans have shown a 1.15 × 10⁻⁶ M plasma concentration of rosmarinic acid following a single oral dose of an herbal extract (i.e., *Perilla frutescens* leaves extract) containing 200 mg of rosmarinic acid (Baba et al., 2005).

Persuasive evidence suggests that oxidative stress contributes to the neurodegenerative process in AD (Butterfield et al., 2002). Oxidative stress reflects a situation wherein ROS, such as free radicals and their products, are in excess over the antioxidant defense system. Aβ significantly increases superoxide and peroxynitrite production and enhances membrane lipid peroxidation before apoptotic cell death (Forman et al., 2004). Indeed, increased peroxynitrite formation and membrane lipid peroxidation are directly associated with degenerating neurons in AD patients, suggesting that peroxynitrite-induced lipid peroxidation may play a key role in the cell death process induced by Aβ in AD (Butterfield et al., 2002). Previous investigators have shown that rosmarinic acid reduced iron-dependent anthracycline induced lipid peroxidation of rat cardiomyocytes (Chlopcikova et al., 2004), inhibited the hemolysis of rat erythrocytes induced by hydrogen peroxide (Liu et al., 1992), and attenuated ROS production induced by toxins in human hepatoma cell line (Renuzzi et al., 2004). In the present study, we have shown that rosmarinic acid reduced, in a concentration-dependent manner, Aβ42-induced ROS formation and lipid peroxidation, thus suggesting that this natural compound is a novel and effective neuroprotective agent against oxidative damage induced by Aβ. Because antioxidants are known to attenuate Aβ-induced oxidative injury (Cash et al., 2002), it is likely that the antioxidant properties of rosmarinic acid could contribute to its beneficial effect. Interestingly, Ono et al. (2004) have recently shown that rosmarinic acid inhibited the formation of fibrils from Aβ and destabilized preformed Aβ fibrils in vitro. Therefore, in our PC12 cells, rosmarinic acid may inhibit ROS production directly or indirectly by preventing fibril formation from Aβ.

Oxidative stress, via the MAP kinase pathway, leads to tau protein hyperphosphorylation in AD (Puig et al., 2004). A consequence of tau hyperphosphorylation in AD is a reduction in its ability to bind microtubules and to promote microtubule assembly, a destabilization of microtubule network, and ultimately, NFT formation and neuronal death (Nordberg, 2004). Tau protein is the substrate for different oxidative stress-responsive kinases. Among these, p38 MAP kinase (Puig et al., 2004) and GSK-3β (also named tau kinase) (Esposito et al., 2006) are primarily involved in tau hyperphosphorylation. Our results showed that rosmarinic acid is able to inhibit tau hyperphosphorylation, probably acting through the inhibition of p38 MAP kinase pathway but not through the inhibition of GSK-3β hyperphosphorylation. The intimate molecular mechanism of rosmarinic acid at the basis of the selective p38 MAP kinase inhibition is still under investigation. Interestingly, other antioxidants, such as vitamin C, are able to inhibit p38 MAP kinase activity (Pearl-Yafe et al., 2004) but failed to inhibit GSK-3β activation induced by Aβ (Esposito et al., 2006). This observation highlights that, although oxidative stress is the pivotal event in the activation of both pathways, it is not uniquely responsible for these events.

Experimental studies have shown that Aβ induces tau hyperphosphorylation in a number of cell types, including primary septal cultures (Zheng et al., 2002), rat cortical neuronal cultures (Alvarez et al., 1999), and human neuroblastoma cells. In the present study, we have shown for the first time that Aβ42 induced tau hyperphosphorylation in PC12 cells and, more importantly, that rosmarinic acid reduced such abnormal changes by acting on p38 MAP kinase pathway.

A strong correlation exists between Aβ-induced oxidative stress and neurotoxicity (Esposito et al., 2006). Cell death exhibits typical features of apoptosis, such as membrane blebbing, chromatin condensation, and DNA fragmentation (Stein and Johnson, 2003). Apoptosis is associated with the activation of a family of aspartic acid-specific cysteine proteases, referred to as caspases; among these, the activation of pro-caspase-3 to caspase-3 is a central event in the execution phase of apoptosis (Nicholson and Thornberry, 1997). The Aβ peptide has been shown to induce apoptosis in neurons, including PC12 cells, which may be responsible for neuronal death in AD (Martin et al., 2001). In the present study, we have shown that rosmarinic acid inhibited caspase-3 activation and DNA fragmentation, thus suggesting that rosmarinic acid could affect the execution phase of Aβ-induced apoptosis.

These results are consistent with the ability of rosmarinic acid to decrease the apoptosis induced by aflatoxin B1 in a human hepatoma cell line (Hep G2) (Renuzzi et al., 2004), as well as by hydrogen peroxide in astrocytes. The protective effect of rosmarinic acid on apoptosis could be because of, at least in part, its antioxidant properties and its ability to inhibit lipid peroxidation as described above. Conversely, others have shown that rosmarinic acid induces p56lck-dependent apoptosis in Jurkat and peripheral T cells (Hur et al., 2002).

Based on the present results and those found in the literature, a hypothetical series of events that leads to cell death may be as follows. Rosmarinic acid inhibits ROS formation and hence lipid membrane peroxidation, resulting in a significant inhibition of Aβ-dependent oxidative stress neurotoxicity and apoptosis. In addition, rosmarinic acid, by decreasing ROS production during the early phase of Aβ insult, may inhibit p-p38 MAP kinase, which in turn hyperphosphorylates tau protein, thus preventing the formation of NFT.

In conclusion, the data presented here are the first to show the cytoprotective effect of sage against Aβ toxicity in neuronal cells, which may provide the pharmacological basis underlying the traditional use of this spice in the treatment of...
AD. Rosmarinic acid could contribute, at least in part, to sage-induced neuroprotective effects because this natural compound exerts neuroprotective, antioxidative, and anti-apoptotic effects against AB insult. Finally, the present study opens the eventuality to explore the possible use of rosmarinic acid, a very low toxic natural compound (Parnham and Kesselring, 1985) as a therapeutic approach in AD.

References


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