ORIGINAL ARTICLES

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Extracts of *Cynomorium songaricum* protect human neuroblastoma cells from β-amyloid$_{25-35}$ and superoxide anion induced injury

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In Traditional Chinese Medicine a number of herbs are used to alleviate age-related diseases including memory impairment and dementia, among them stems of *Cynomorium songaricum*, Cynomoriaceae. In this study, we evaluated the protective effect of different extracts of aerial parts of *C. songaricum* on amyloid-β peptide (Aβ) and hypoxanthine/xanthine oxidase induced cell death in SK-N-SH neuroblastoma cells. Aβ (20 μM) as well as superoxide anions generated by the hypoxanthine/xanthine oxidase system both reduced cell viability to about 60%. The methanolic extract of *C. songaricum* attenuated Aβ induced cell death at concentrations of 100 and 10 μg/ml, an even stronger effect was observed for the ethyl acetate fraction obtained from the crude methanolic extract. On the other hand, the dichloromethane as well as water fractions showed no protective effects. In order to further analyze the protective mode of action, the ability of extracts to protect against superoxide anions induced cell death was also evaluated. In this system, cell viability could again be restored by methanol and ethyl acetate extracts, the latter showing significant protective effects even at concentrations as low as 0.1 μg/ml.

1. Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder associated with a global impairment of higher mental function, and presenting an impairment of memory as the cardinal symptom (Bartus et al. 1982). Histopathological hallmarks of the disease are the extracellular deposition of amyloid-β peptide (Aβ) in senile plaques, the appearance of intercellular neurofibrillary tangles (NFT), a loss of cholinergic neurons, and extensive synaptic changes in the cerebral cortex, hippocampus and other areas of brain essential for cognitive functions.
To date, the cause and the mechanism by which neurons die in AD remain unclear, but Aβ has been established as a crucial factor in AD pathogenesis. Aβ deposition may cause neuronal death via a number of possible mechanisms, including oxidative stress, excitotoxicity, energy depletion, inflammation and apoptosis. Despite this multifactorial etiology, genetics plays a key role in disease progression. However, environmental factors (e.g. cytokines, neurotoxins) may be even more important in the development and progression of AD. Several lines of evidence support the involvement of oxidative stress (Behl and Moosmann 2002). Oxidative damage, mediated by reactive oxygen species (ROS) generated following cell lyses, oxidative bursts, or an excess of free transition metals, has been hypothesized to play a pivotal role in AD neurodegeneration (Christen 2000). On the other hand, postmortem studies provide direct morphological and biochemical evidence that some neurons in the AD brain degenerate via an apoptotic mechanism, which may or may not be linked to ROS (Eckert et al. 2003). The biochemical mechanism underlying the formation of AD is clearly complex, with many factors contributing to the neuropathology. Thus it is not surprising that a number of different intervention therapies are currently being researched to address distinct aspects of disease.
Cynomorium songaricum Rupr. (Cynomoriaceae) is an achlorophyllous holoparasite that is distributed in the north-western part of China. Among its chemical constituents, steroids, triterpenes, flavonoids and lignans have been reported previously (Chu et al. 2006; Jiang et al. 2001; Ma et al. 1992). According to the ancient Chinese medical literature, *C. songaricum* is effective against symptoms of aging – ranging from mild forms of memory impairment to dementia –, regulating endocrinopathy and improving sexual function (Qi 1999; Ma et al. 2000). As different extracts of this plant recently proved to be able to protect cells against staurosporine-induced neurotoxicity (Lu et al. 2009), the purpose of this study was to evaluate its ability to attenuate the Aβ$_{25-35}$-induced neurotoxicity in a cell based assay. Aβ$_{25-35}$ has been identified as the toxic fragment of the human amyloid-β$_{1-42}$ (Pike et al. 1995) and is able to induce toxicity in neuronal cell lines. In order to further analyze the protective mode of action, the ability of extracts to protect against superoxide anions (hypoxanthine/xanthine oxidase system) induced cell death was also evaluated.

2. Investigations, results and discussion

Peptides derived from the human amyloid-β sequence were shown to induce toxicity in neuronal cell lines, primary cultures of neuronal origin as well as *in vivo* (Pike et al. 1993; Zhang et al. 1994). In order to examine a possible neuroprotective effect of different extracts of *C. songaricum*, we determined whether cell death induced by $A\beta_{25-35}$, which has been identified as the toxic fragment of the human amyloid-β_{1-42} (Pike et al. 1995), in human neuroblastoma SK-N-SH cells could be attenuated. Cell viability in the presence of 20 μM $A\beta_{25-35}$ was significantly reduced to 58% compared to untreated control (Fig. 1). Cytotoxicity studies conducted with *C. songaricum* extracts confirmed that they did not affect cell survival at concentrations ≤100 μg/ml. Thus, the neuroprotective effect of extracts was assayed at concentrations of 100, 10, 1 and 0.1 μg/ml. Figure 1A shows that the MeOH-extract of *C. songaricum* at 100 and 10 μg/ml significantly protected the cells against $A\beta_{25-35}$-induced cell death with 100 μg/ml recovering the cell viability to about 85%. Obviously, active compounds could be concentrated in the EtOAc fraction which led to a nearly 100% cell recovery at a concentration of 100 μg/ml and showed pronounced protective effects also at 10 μg/ml (Fig. 1B). On the other hand, the CH_2Cl_2 fraction and the remaining aqueous phase did not show distinct protective effects against $A\beta_{25-35}$ induced cell injury at all tested concentrations (Fig. 1 C/1D).

Neuronal death in the brains of AD patients seems to be associated with increased oxidative stress and previous investigations have shown that reactive oxygen species can trigger neuronal death (Pappolla et al. 1997; Pike et al. 1993). Among the major reactive oxidants in cells are superoxide anions which can be produced by the hypoxanthine/xanthine oxidase system (Beyer et al. 2003). Hypoxanthine and xanthine are generated as products of the catabolism of adenosine triphosphate and the purin nucleosides and thus are important biological sources of reactive oxygen species (Benoit and Taylor 1997). In order to further evaluate the possible protective mode of action of *C. songaricum* extracts, their ability to protect SK-N-SH cells against an insult induced by the hypoxanthine/xanthine oxidase system was analyzed. Under the chosen experimental conditions, incubation of the cells with 100 μM hypoxanthine and 2 mU/mL xanthine oxidase for 30 min led to a reduction in cell viability of about 40% compared to untreated control (Fig. 2). As shown in Fig. 2A and 2B, also in this assay, viability could be restored by the MeOH and EtOAc extracts with significant protective effects even at concentrations as low as 1 and 0.1 μg/ml (EtOAc extract).

Several lines of evidence have now converged to suggest that increased oxidative stress and disturbed defense mechanisms in the brain of AD patients might result in a self-programming cascade of neurodegenerative events (Markersbery 1997). Over-production of Aβ has been proposed as one cause of AD. Some studies showed that Aβ mediated toxicity on cell-lines can be prevented by vitamin E and other antioxidants (Yatin et al. 2000). Another study showed that hydrogen peroxide is involved in Aβ mediated toxicity (Atwood et al. 2003). These results suggest a close relationship between the amyloid-β induced cell death and oxidative stress. Consistent with these findings, MeOH and EtOAc extracts of *C. songaricum* that were able to attenuate the $A\beta_{25-35}$ induced toxicity on human neuroblastoma cells also proved to be strongly active in a cell model where toxicity was induced by oxidative stress. Thus, their mode of action might be closely related with this antioxidative effect.

In conclusion, our results show that extracts of *C. songaricum*, a plant that is used in China since ancient times to treat age-related neuronal diseases such as dementia, possess neuroprotective effects in vitro, thus supporting the traditional use. Further studies in order to identify active compounds are under way. Investigating the neuroprotec-

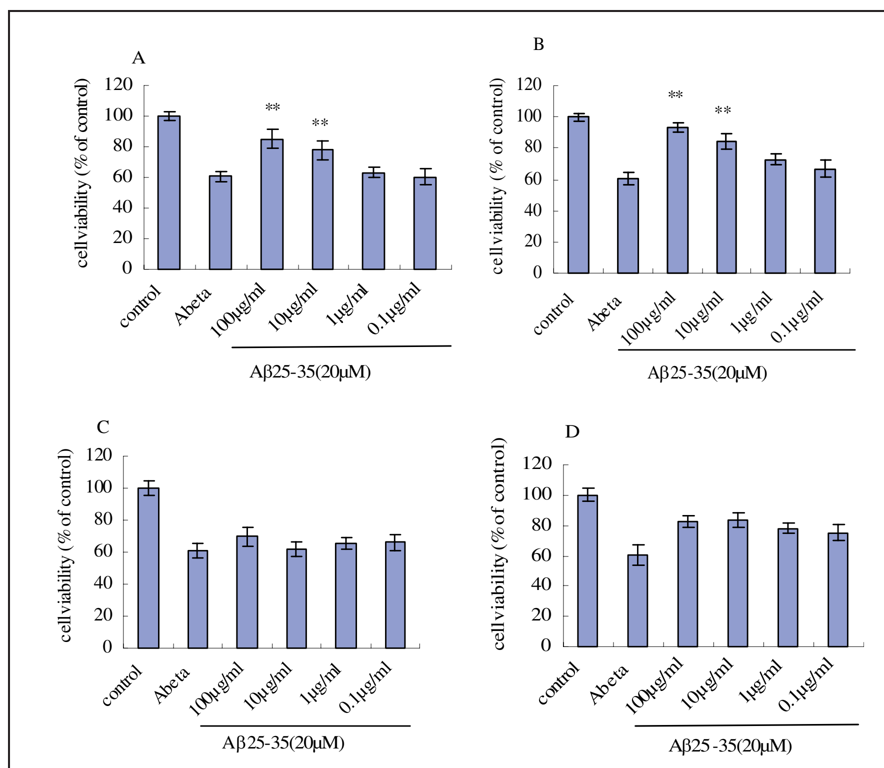

Fig. 1:
Protective effects of four extracts of *C. songaricum* (A: MeOH-extract, B: EtOAc-extract, C: CH_2Cl_2-extract, D: H_2O-extract) against $A\beta_{25-35}$ (20 μM) induced toxicity on SK−N−SH cells. The cells were treated with different concentrations of extracts (100, 10, 1, 0.1 μg/ml) and $A\beta_{25-35}$ (20 μM). The cell viability was determined by the MTT assay after 96 h as indicated. Means ± SD of at least three experiments are shown. $^{**}p < 0.01$ when compared with controls

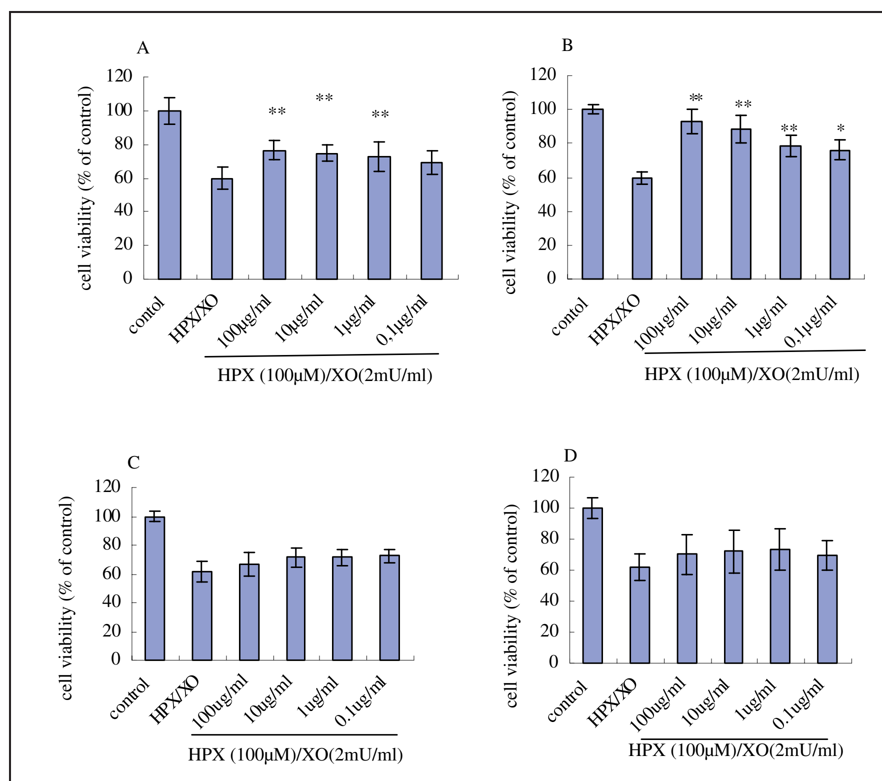

Fig. 2:
Protective effects of four extracts of *C. songaricum* (A: MeOH-extract, B: EtOAc-extract, C: CH_2Cl_2-extract, D: H_2O-extract) against hypoxanthine (100μM)/xanthine oxidase (2 mU/ml) (HPX/XO) induced toxicity on SK−N−SH cells. The cells were treated with different concentrations of extracts (100, 10, 1, 0.1 μg/ml) and hypoxanhine/xanthine oxidase for 0.5 h. The cell viability was determined after 24 h by the MTT assay as indicated. Means ± SD of at least three experiments are shown. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with controls

tive effects of extracts and constituents of *C. songaricum in vitro* and *in vivo* may provide insights into novel therapeutic strategies for Alzheimer's and other neurodegenerative diseases.

3. Experimental

3.1. Chemicals

$A\beta_{25-35}$ was obtained from Bachem, Weil am Rhein, Germany. Hypoxanthine, xanthine oxidase and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma, Taufkirchen, Germany. The cell culture media were supplied by Biochrom, Berlin, Germany.

3.2. Plant material

The Chinese medicine *Cynomorium songaricum* (Ch.-B.: 040801h349) used for this study was purchased from Herbasin Hilsdorf GmbH, Rednitzhembach, Germany.

3.3. Preparation of extracts

Dried and ground aerial parts of *C. songaricum* (50 g) were extracted three times with 300 ml MeOH (each for 1 h). The crude extracts were evaporated under reduced pressure to give a viscous residue (MeOH-extract, 6.6 g). The residue was re-suspended in 100 ml water and successively extracted with CH_2Cl_2 and EtOAc to give CH_2Cl_2-(yield: 10.9%) and EtOAc-extracts (yield: 3.6%). The remaining aqueous solution was also evaporated under reduced pressure (H_2O-extract). All residues were solved in 0.2% aq. DMSO at required concentrations for the different bioassays.

3.4. Cell culture

SK-N-SH human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, No. HTB-11). Cells were routinely cultivated in minimal essential medium (MEM + Earl's salts) containing sodium pyruvate, non-essential amino acids and 10% fetal calf serum at 37 °C under 5% CO_2 in a moisture-saturated atmosphere.

3.5. Cytotoxicity assay

SK-N-SH cells were seeded at a density of 1×10^4 cells/well in 96 well culture plates. After 24 h, extracts of *C. songaricum* at final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.8 μg/ml were added and incubated for 96 h. Then the cell viability was determined by measuring the cell's potential to reduce MTT to MTT-formazan (Mosmann 1983). Briefly, 20 μl of MTT stock solution in PBS (5 mg/ml) were added to each well and incubated at 37 °C for 2 h. Then the formed formazan was dissolved in DMSO. Optical density of the resulting solution was colorimetrically determined at 580 nm (reference wavelength 620 nm) using a microplate reader (Tecan). The dose-effect curve for each test compound was linearized by regression analysis and used to derive the IC_{50} values.

3.6. Determination of the ability to protect SK-N-SH cells against $A\beta_{25-35}$ insult

$A\beta_{25-35}$ was firstly dissolved in PBS at a concentration of 1 mM, then incubated at room temperature for approximately 24 h and at 37 °C for about 7 days to form aggregated Aβ, and then stored at 4 °C until use (Chiou et al., 2006).
SK-N-SH cells were seeded at a density of 1.5×10^4 cells/well in 96 well culture plates. 24 h after plating, the cells were treated with extracts of *C. songaricum* at final concentration of 100, 10, 1 and 0.1 μg/ml in serum-free MEM containing 1% human albumin. 4 h later, $A\beta_{25-35}$ (20 μM) was added. After incubation for 96 h, cell viability was again determined by the MTT assay.

3.7. Determination of the ability to protect SK-N-SH cells against superoxide anion induced insult

SK-N-SH cells were seeded at a density of 1×10^4 cells/well in 96 well culture plates. After 24 h, the medium was removed and the cells were exposed to 100 μM hypoxanthine and 2 mU/ml xanthine oxidase in PBS (Beyer et al. 2003, 2005). Extracts of *C. songaricum* at final concentrations of 100, 10, 1 and 0.1 μg/ml were added and incubated for 0.5 h. Then the supernatant was removed and the cells were cultivated in MEM + Earl's salts supplemented with 10% fetal calf serum for 24 h. Cell viability was determined by reduction of MTT using DMSO to dissolve the purple formazan as descried above.

3.8. Statistical analyses

Data are expressed as mean ± SD of at least three separate experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. A value of $p < 0.05$ was considered statistically significant.

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