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Impact of plant extracts tested in attention-deficit/hyperactivity disorder treatment on cell survival and energy metabolism in human neuroblastoma SH-SY5Y cells

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3 **Impact of plant extracts tested in attention-deficit/hyperactivity disorder**
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6 **treatment on cell survival and energy metabolism**
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9 **in human neuroblastoma SH-SY5Y cells**
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Abstract

Plant extracts like *Hypericum perforatum* and Pycnogenol[®] have been tested as alternatives to the classical ADHD drugs. It has been possible to describe neuroprotective effects of such plant extracts. A reduction of ADHD symptoms could be shown in clinical studies after the application of Pycnogenol[®], which is a pine bark extract. The impacts of the standardized herbal extracts *Hypericum perforatum*, Pycnogenol[®] and Enzogenol[®] up to a concentration of 5.000 ng/ml on cell survival and energy metabolism in human SH-SY5Y neuroblastoma cells has been investigated in the present examination. *Hypericum perforatum* significantly decreased survival of cells after treatment with a concentration of 5000 ng/ml, whereas lower concentrations exerted no significant effects. Pycnogenol[®] induced a significant increase of cell survival after incubation with a concentration of 32.25 ng/ml and a concentration of 250 ng/ml. Other applied concentrations of Pycnogenol[®] failed to exert significant effects. Treatment with Enzogenol[®] did not lead to significant changes in cell survival.

Concerning energy metabolism, treatment of cells with a concentration of 5000 ng/ml *Hypericum perforatum* led to a significant increase of ATP levels, whereas treatment with a concentration of 500 ng/ml had no significant effect. Incubation of cells with Pycnogenol[®] and Enzogenol[®] exerted no significant effects.

None of the tested substances caused any cytotoxic effect when used in therapeutically-relevant concentrations.

Keywords: Pycnogenol[®], Enzogenol[®], *Hypericum*, cell survival, energy metabolism, neuroblastoma cells

Introduction

Over decades, the standard pharmacotherapy of attention-deficit/hyperactivity disorder (ADHD) had comprised psychostimulants like methylphenidate and amphetamine as well as the selective norepinephrine reuptake inhibitor atomoxetine later on. Since some patients do not respond to the classical ADHD drugs or show side effects, the use of herbal extracts like *Hypericum perforatum* or Pycnogenol[®] (*Pinus pinaster*) as supplementation or as monotherapy has been discussed in the last few years. Concerning combinations of extracts, the results of a study by Lyon et al. (2001) suggest a possible improvement of ADHD symptoms after a supplementation with an extract combination of *Panax quinquefolium* and *Ginkgo biloba*.

The extract of *Hypericum perforatum* is known to exert both antioxidative properties and protective effects (e.g. Benedi et al., 2004; Breyer et al., 2007; El-Sherbiny et al., 2003; Sanchez-Reus et al., 2007). However, no improvement of ADHD symptoms after a treatment of eight weeks has been found in a recently published randomised, double-blind, placebo-controlled study (Weber et al., 2008). The patients had not received any other drugs during this study.

A plant extract which has been discussed extensively in terms of ADHD treatment over the last few years is Pycnogenol[®]. Pycnogenol[®] stands for a bark extract of the French maritime pine (*Pinus pinaster*) and mainly consists of procyanidines as well as catechins and phenolic acids (Packer et al., 1999). The antioxidant activities of this extract have been described. It could be shown in vitro that Pycnogenol[®] is a free radical scavenger (Packer et al., 1999), that it stimulates activities of antioxidant enzymes like Cu, Zn superoxide dismutase (Fitzpatrick et al., 1998) and that it influences the glutathione metabolism (Berryman et al., 2004). Furthermore, protective effects of Pycnogenol[®] on neuronal cells in context with toxic insults like acrolein (Ansari et al., 2008), β -amyloid (Peng

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3 et al., 2002), ethanol (Siler-Marisiglio et al., 2004) or glutamate (Kobayashi et al., 2000) have
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6 been shown.

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8 These characteristics of Pycnogenol[®] may be important factors for the treatment of
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10 ADHD, because indices for reactive oxygen species induced oxidative stress have been
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12 discussed in children and young adults, in part controversially (Antalis et al., 2006;
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14 Chovanova et al., 2006; Joshi et al., 2006; Ross, et al., 2003; Spahis et al., 2008) as well as in
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16 adults suffering from ADHD (Bulut et al., 2007; Selek et al., 2008).
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20 Chovanova et al. (2006), measuring with comet assay, have found a significantly
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22 increased level of oxidative DNA damage in children with ADHD in comparison to healthy
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24 controls. Spahis et al. (2008) have described significantly reduced plasma malone dialdehyde
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26 levels in ADHD children, whereas Ross et al. (2003) have found significantly increased
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28 ethane levels in the alveolar breath of children with ADHD. Ethane is a non-invasive marker
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30 of oxidative damage to n-3 fatty acids. On the other hand, Joshi et al. (2006) have found no
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32 significant alterations of lipid peroxidation products in the plasma of children with ADHD
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34 compared to controls, while Antalis et al. (2006) have detected no significant differences of
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36 urinary isoprostanes comparing a young adult ADHD population with controls.
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42 In order to investigate alterations of the antioxidant vitamin status in ADHD, Spahis et
43
44 al. (2008) have measured the plasma levels of retinal, β -carotene and vitamin E in children
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46 with ADHD. The two vitamin E forms, α - and γ -tocopherol, have been found to be
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48 significantly higher in ADHD children compared with control subjects, whereas retinol and
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50 β -carotene contents showed no modifications.
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54 Regarding adult patients, Bulut et al. (2007) have described significantly elevated
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56 malondialdehyde plasma levels, as a product of lipidperoxidation, which is induced by
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58 reactive oxygen species. In a recently published study, Selek et al. (2008) could show a
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60 significantly reduced activity of total Cu, Zn superoxide dismutase as well as significantly
enhanced levels of oxidant nitric oxide in the serum of adult patients with ADHD.

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3 Aiming at evaluating the effects of Pycnogenol[®] on ADHD symptoms, Trebaticka et
4 al. (2006) could demonstrate in a randomised, placebo-controlled, double blind study, that a
5 supplementation with Pycnogenol[®] for one month had caused a significant reduction of
6 hyperactivity in children with ADHD, had improved attention and visual-motoric
7 coordination and their ability to concentrate. One month after the treatment had finished, a
8 relapse of symptoms had been observed. On the other hand, a study with adult patients had
9 shown no reduction of ADHD symptoms after treatment with Pycnogenol[®] (Tenenbaum et
10 al., 2002).
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22 An extract similar to Pycnogenol[®] is Enzogenol[®], which is a bark extract of the New
23 Zealand pine *Pinus radiata* containing flavonoids. In combination with vitamin C, Enzogenol[®]
24 has improved the cognitive performance in older individuals (Pipingas et al., 2008). Vitamin
25 C alone has failed to reach this effect. Previous studies (e. g. Young et al., 2006) which have
26 been using this combination have indicated improvements of oxidative stress parameters.
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34 Also Ginko biloba has been shown to be effective for treating ADHD however being
35 less effective than methylphenidate (Niederhofer, 2010, Salehi et al., 2010).
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39 Summarizing the studies cited above, only little is known about the effects of plant
40 extracts on cell metabolism. Therefore, the aim of the present study was the examination of
41 effects of both pine bark extracts and *Hypericum perforatum* on cell survival of human
42 neuronal (SH-SY5Y) and on energy metabolism.
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50 **Materials and Methods**

51 *Preparation and incubation of cells*

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53 Human SH-SY5Y neuroblastoma cells were cultured in a 5% CO₂ atmosphere in heat-
54 inactivated Roswell Park Memorial Institute medium (RPMI) (Gibco/BRL, Eggenstein,
55 Germany), supplemented with 15% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1%
56 penicillin-streptomycin and 1% glutamine. The *Hypericum perforatum* extract was a kind gift
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3 from Casella-med GmbH & Co. KG (Köln, Germany). The pine bark extracts were obtained
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5 from the Department of Child and Adolescent Psychiatry at the Albert-Ludwigs-University of
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7 Freiburg. All drugs were dissolved in dimethylsulfoxide and concentrations of 31.25, 62.5,
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9 125, 250, 625, 1250, 2500 and 5000 ng/ml were applied. A respective dissolvent was
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11 applied for controls. Each concentration was tested by a minimum of three single
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13 experiments.
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20 *Measurement of metabolic activity and survival*

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22 Cells were exposed to drugs in the above-mentioned concentrations for 24 h at a
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24 temperature of 37° C, using 96-well dishes with 36,000 per well. The survival of cells was
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26 determined by a biochemical assay using a modified tetrazolium method (EZ4U, Biozol,
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28 Eching, Germany). This test is based on the ability of living cells to transform colourless
29
30 tetrazolium salts into deeply coloured formazans by mitochondrial dehydrogenases. Cell
31
32 viability was quantified by photometric determination of formazan-like products with an
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34 ELISA-reader (Dynex, Stuttgart, Germany).
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41 *Measurement of ATP content*

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43 36,000 cells per well were plated into 96-well dishes and exposed to the tested drugs
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45 in concentrations of 500 and 5000 ng/ml for 24 h at a temperature of 37°C. The ATP content
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47 of cells was determined by using an ATP Bioluminescence Assay (Boehringer, Ingelheim,
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49 Germany).
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53 The amount of ATP was measured by using a microplate scintillation counter
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55 “TopCount” (Perkin Elmer, Ueberlingen, Germany), which allows for quantitative
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57 measurement via luminescence which is detected by single photon counting.
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Statistical analysis

Data are expressed in a percentage of respective controls and are displayed as mean values \pm standard error of the mean (S.E.M.).

In order to evaluate the effects of treatment, Kruskal Wallis tests were used to determine differences among the treatment groups. In case of significance, Dunn's method was performed. The level of significance was set to $P < 0.05$. Statistical evaluation was carried out using SigmaStat software (Jandel Scientific, Erkrath, Germany).

Results

Effects of plant extracts on survival of SH-SY5Y cells

The impact of the plant extracts Hypericum perforatum, Pycnogenol[®] and Enzogenol[®] on cell survival was tested in human neuroblastoma SH-SY5Y cells in concentrations ranging from 31.25 to 5000 ng/ml. The treatment of cells with Hypericum perforatum in concentrations of 5000 ng/ml ($90 \pm 2 \%$, $p < 0.05$) has significantly decreased the survival of cells (Fig.: 1). The treatment of cells with lower concentrations of Hypericum perforatum has exerted no significant effects (Fig.: 1). Pycnogenol[®] has induced a significant increase of cell survival after its incubation in concentrations of 32.25 ng/ml ($112 \pm 2 \%$, $p < 0.05$), 125 ng/ml ($113 \pm 3 \%$, $p < 0.05$) and 250 ng/ml ($113 \pm 2 \%$, $p < 0.05$), while concentrations of 62.5 ng/ml, 625 ng/ml ($102 \pm 2 \%$), 1250 ng/ml ($104 \pm 2 \%$), 2500 ng/ml ($101 \pm 2 \%$) and 5000 ng/ml ($96 \pm 2 \%$) have failed to exert significant effects (Fig.: 2). Treatment of SH-SY5Y cells with Enzogenol[®] has not led to significant changes in cell survival (Fig.: 3).

Effects of plant extracts on energy metabolism of SH-SY5Y cells

The impact of plant extracts on the ATP content of human neuroblastoma cells was measured by using concentrations of 500 and 5000 ng/ml. The incubation of cells with

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3 Pycnogenol[®] (500 ng/ml: $98 \pm 6 \%$, 5000 ng/ml: $90 \pm 7 \%$) and with Enzogenol[®] (500 ng/ml:
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6 $86 \pm 4 \%$, 5000 ng/ml: $98 \pm 7 \%$) has exerted no significant effects (Fig.: 4 and 5), while the
7
8 incubation with Hypericum perforatum in a concentration of 5000 ng/ml (135 ± 9 , $p < 0.05$)
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10 has resulted in a significant increase of ATP levels, whereas using a concentration of 500
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12 ng/ml ($106 \pm 5 \%$) had no significant impact (Fig.: 4 and 5).
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18 Discussion

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20 A group of substances for the treatment of ADHD which have been tested intensively
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22 over the last few years, are plant extracts with antioxidative or neuroprotective capacities like
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24 Pycnogenol[®], Hypericum perforatum or Panax quinquefolium and Ginkgo biloba. Regarding
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26 these extracts, the focus of interest has been on Pycnogenol[®] in the last years. A chemically
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28 similar extract has been Enzogenol[®]. The aim of the present study has laid on evaluating the
29
30 effects of these plant extracts on both cell survival and energy metabolism of human
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32 neuroblastoma SH-SY5Y cells.
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36 The neuroprotective impact of Hypericum perforatum (e.g. Breyer et al., 2007) and
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38 Pycnogenol[®] (e. g. Peng et al., 2002) has been described in the literature. It has to be
39
40 mentioned that in the course of our study, none of the tested substances have exerted any
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42 intense cytotoxic effect. In our study, treatment with Hypericum perforatum in a
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44 concentration of 5000 ng/ml has significantly decreased survival of cells, whereas using lower
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46 concentrations of Hypericum perforatum has exerted no significant effects. The incubation
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48 with Pycnogenol[®], using concentrations of 32.25 ng/ml and 250 ng/ml has induced a
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50 significant increase of cell survival, whereas the incubation with Enzogenol[®] has not led to
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52 any significant reactions at all. Both Benedi et al. (2004) and Breyer et al. (2007) have not
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54 found any significant effects of Hypericum perforatum on neuronal cell survival in
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56 comparable studies, whereas it has been shown that Pycnogenol[®] exerted protective effects
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58 against various neuronal cell insults. Ansari et al. (2008) have detected protective effects of
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3 Pycnogenol[®] following acrolein-induced cytotoxicity in SH-SY5Y cells. It could be shown
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5 that pre-treatment with Pycnogenol[®] in concentrations of 50 and 100 µg/ml) has significantly
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7 reduced the amount of acrolein-induced reactive oxygen species, has reduced superoxide
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9 levels and reduced cell death. Furthermore, an attenuation of the decline of reduced
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11 glutathione has followed the preincubation of cells with Pycnogenol in concentrations of (50
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13 and 100 µg/ml), while a decrease of oxidized glutathione has followed the preincubation of
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15 cells with Pycnogenol[®] in a concentration of 50 µg/ml. Pre-treatment with Pycnogenol[®] in
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17 concentrations of 50 and 100 µg/ml has also reduced the products of oxidative protein and
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19 membranlipid damage induced by acrolein significantly. The exclusive use of Pycnogenol[®] in
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21 concentrations of up to 200 µg/ml has had no effects on cells.
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27 Peng et al. (2002) have examined the neuroprotective effects of Pycnogenol[®] on PC12
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29 cells, describing the prevention of A β ₂₅₋₃₅-induced losses of cell viability after pre-treatment
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31 with Pycnogenol[®] in concentrations of 20-60 µg/ml. Furthermore, they have described a
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33 significantly decreased number of apoptotic cells after pre-treatment with Pycnogenol[®] in
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35 concentrations of 10-60 µg/ml Pycnogenol[®] as well as a dose-dependent reduction of caspase-
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37 3 activity. The neuroprotective effects of Pycnogenol[®] against the cytotoxicity of ethanol in
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39 cerebral granule cells, including the reduction of reactive oxygen species' products and the
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41 effects on antioxidative enzyme activities, has been shown by Silar-Marsiglio et al. (2004) by
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43 using Pycnogenol[®] in a concentration of 50 µg/ml.
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48 Used in concentrations ranging from 20 to 100 µg/ml, neuroprotective effects of
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50 Pycnogenol[®] could be observed, whereas no impacts on cell survival could be observed in
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52 this concentration range. On the other hand, our study has demonstrated that Pycnogenol[®] led
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54 to a significant enhancement of cell survival at lower concentrations (31.25, 125 and 250
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56 ng/ml).
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60 Our investigation of the plant extracts Pycnogenol[®] and Enzogenol[®] has shown no
significant effects on energy metabolism when used in concentrations of 500 and 5000 ng/ml.

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3 However, treatment with *Hypericum perforatum* extract in a concentration of 5000 ng/ml has
4 induced a significant increase of ATP content in SH-SY5Y cells. This result has been in line
5 with some other examinations (e. g. Breyer et al., 2007), describing that the extract has been
6 able to counteract the glutamate induced energy loss, suggesting that *Hypericum perforatum*
7 has antioxidative and neuroprotective properties. The concentrations of the drugs which we
8 applied here ranging from 31.25 to 5000 ng/ml were derived from clinical studies. In clinical
9 studies the concentrations of the drugs ranged from 1mg/kg/day to 1g/kg/day.
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20 In summary, the efficiency of plant extracts has been shown in clinical studies of
21 patients with ADHD. Hence, plant extracts seem to be alternatives for patients who do not
22 respond to the classical ADHD treatment with psychostimulants or atomoxetine. In order to
23 identify the plant extracts' exact mechanism of action, further investigations on the
24 components of oxidative stress and apoptotic parameters will be mandatory. There exists a
25 theory on the mechanism of action of psychostimulants, stating that reduced dopamine
26 contents in cells lead to decreased contents of both hydrogen peroxide and other products of
27 oxidative stress, as a lowered number of dopamine is deaminated by monoaminooxidase.
28 These parameters would be of particular interest in terms of possible relations to
29 psychostimulants.
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46
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54 **Conflict of Interest**

55
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57
58

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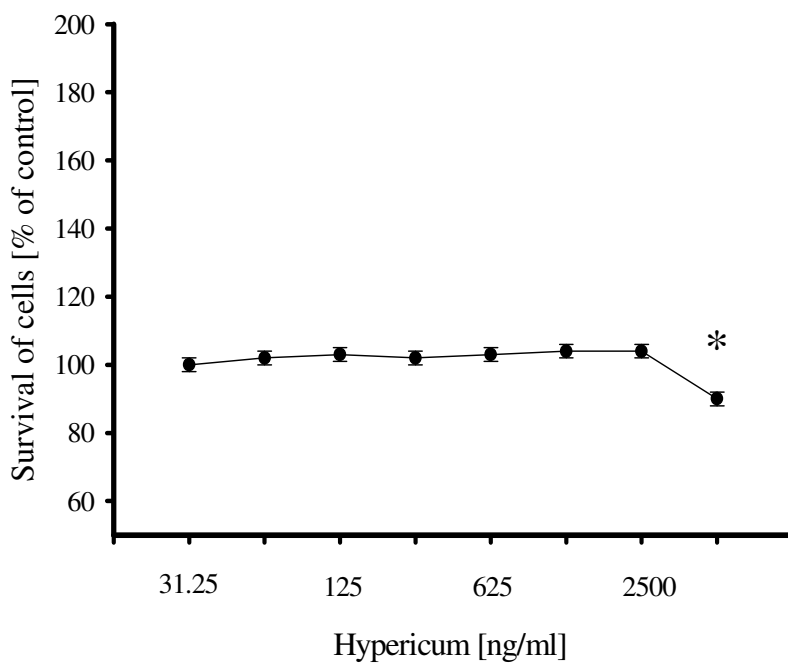


Figure1: Effects of Hypericum perforatum extract on cell survival of SH-SY5Y cells

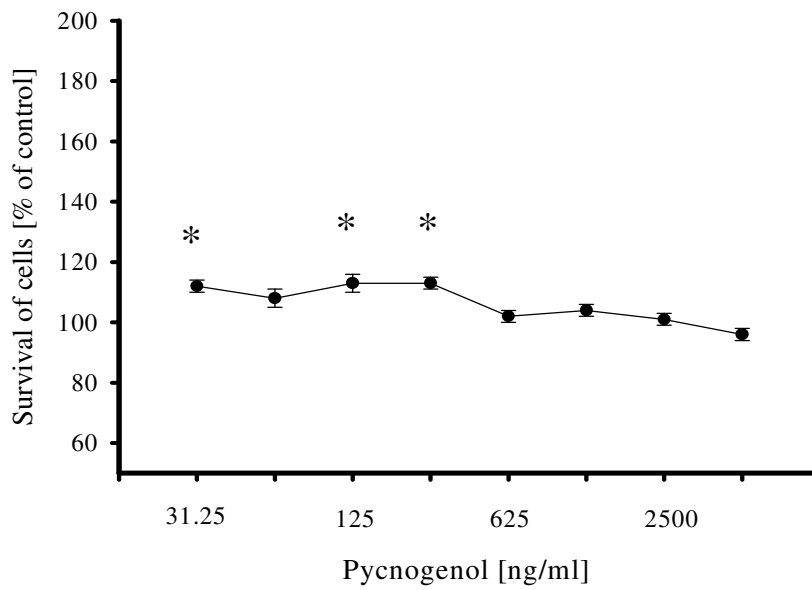


Figure 2: Effects of Pycnogenol[®] on cell survival of SH-SY5Y cells

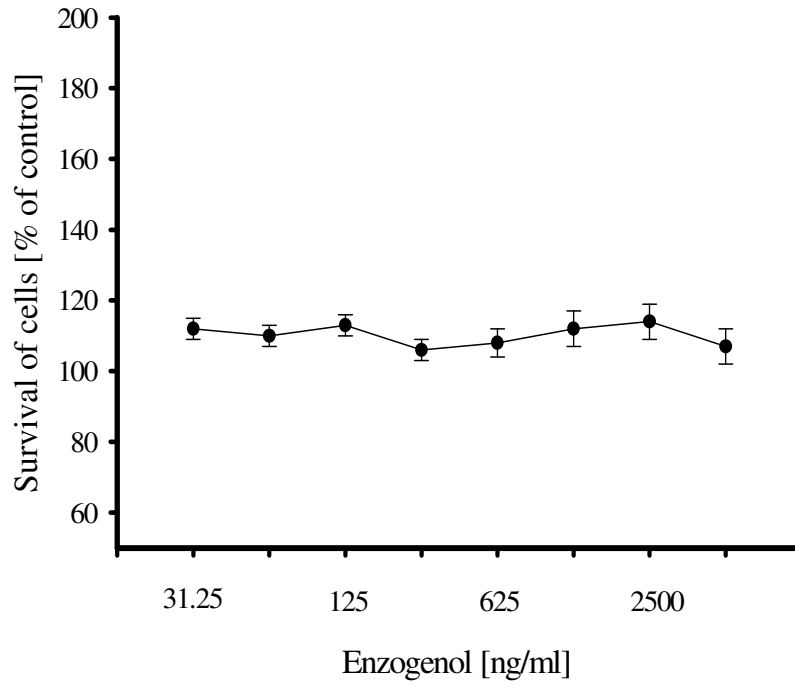


Figure 3: Effects of Enzogenol[®] on cell survival of SH-SY5Y cells

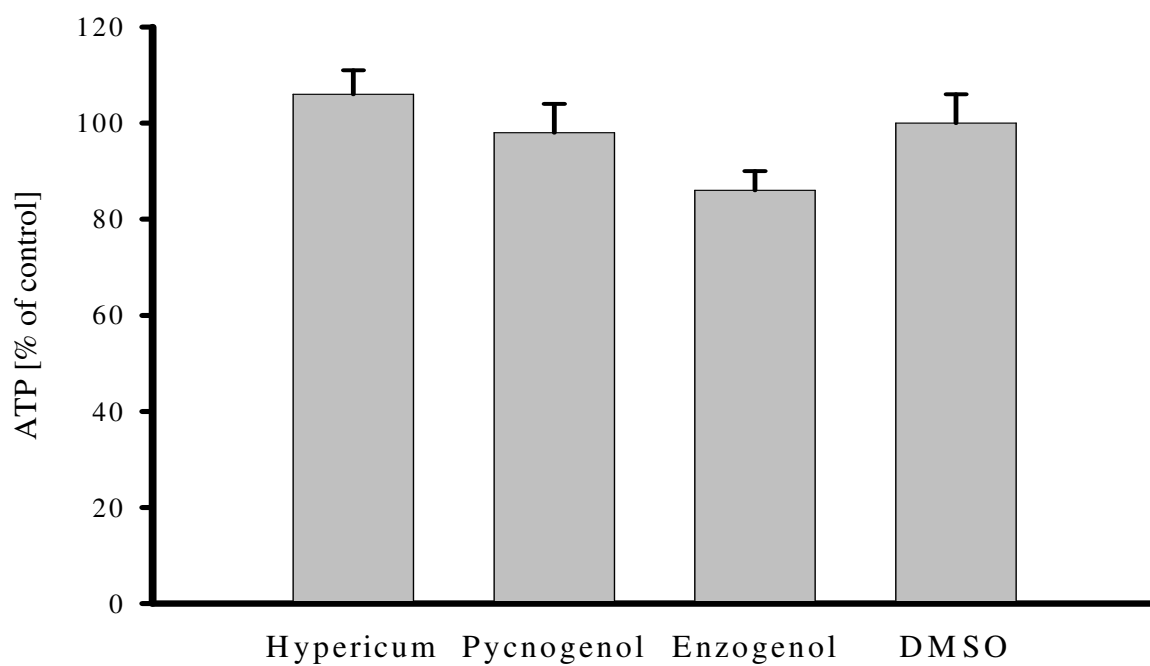


Figure 4: Effects of 500 ng/ml plant extract on ATP content of SH-SY5Y cells

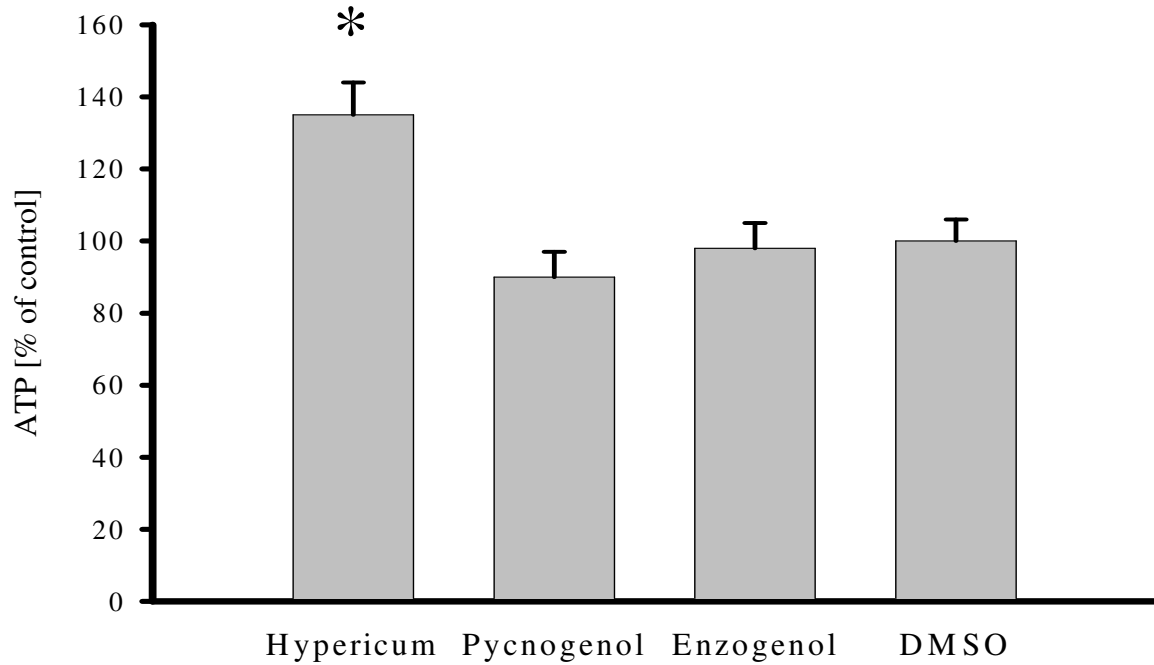


Figure 5: Effects of 5000 ng/ml plant extract on ATP content of SH-SY5Y cells