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Apoptotic-induced Effects of *Castanea sativa* Bark Extract in Human SH-SY5Y Neuroblastoma Cells

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Castanea sativa Mill. wood extract (ENC) has health-promoting qualities such as antibacterial, antiviral, antispasmodic, cardio- and neuroprotective-activities. Its potential towards cancer cells of the central nervous system, however, is still unexplored. This study investigates the apoptotic-enhancing effect of ENC in human neuroblastoma SH-SY5Y cell line. This extract irreversibly decreased cell viability with IC_{50} of 142.8±20.2, 109.3±16.6 and 82.5±8.6 µg/mL for 24h, 48 and 72h of treatment, respectively. This was accompanied by cell shrinkage and tendency to round-up. A concentration-dependent increase in sub G0/G1 cells as well as a rise in cells with nuclear fragmentation and chromatin condensation, both typical of apoptosis, was also observed. In conclusion, the present findings provide initial data on the potential of ENC for neuroblastoma treatment and offer the rationale for further studies aimed at elucidating the full mechanism(s) underlying its effects.

Keywords: *Castanea sativa*, Apoptosis, Neuroblastoma, SH-SY5Y cells, Tannins, Vescalagin, Castalagin, Ellagic acid.

Castanea sativa Mill. belongs to the *Fagacea*e family and it is one of the most spread chestnut species. The nuts are used for human and animal feed, while the wood is used for furniture. Leaves are a source of phenolic bioactive compounds and are used in many ethnobotanic formulations against colds, coughs, diarrhea, and high blood cholesterol, while the flowers are used in beverages like tea [1]. The wood extract, rich hydrolysable tannins, is a plant preparations potentially useful as substitute for nutritive antibiotics [2]. In the past, tannins have been considered as anti-nutritive secondary plant metabolites, which precipitate proteins, inhibit digestive enzymes and affect the utilization of vitamins and minerals, thus causing chronic or subacute effects [3]. Due to their great structural diversity, however, some tannins are commonly used as antihelmintic, antimicrobial and antiviral agents and as supportive treatment for diarrhea [4]. Furthermore, ellagitannins such as geraniin, ellagic acid, punicalagin which are the main hydrolysable tannins in chestnut wood extract, possess reducing and antioxidant activity [5].

The extract obtained by the bark of *Castanea sativa* Mill. (ENC) contains high amounts of hydrolizable tannins such as vescalagin, castalagin, vescalin, castalin, gallic acid, and ellagic acid, whose qualitative and quantitative chemical characterization has been performed by HPLC-DAD-MS [6]. ENC has been shown to exert antispasmodic action by modulation of cholinergic receptors and calcium channels [7, 8], to act as antioxidant and cytoprotective agent in rat cultured cardiomyocytes [6], to possess antihelmintic, antimicrobial, and antiviral activities, to reduce oxidative stress, as well as to prevent DNA damage after oral administration [2]. Moreover, ENC neuroprotective effects have been recently reported in human astrocytoma and neuroblastoma cells as well as rat brain slices subjected to ischemia-like conditions or treated with glutamate or hydrogen peroxide [9, 10]. Neuroblastoma is the third most common type of childhood cancer and is characterized by high incidence and mortality rate. It is treated with highly toxic chemotherapy, surgery and bone marrow transplantation, but due to the toxic side effects of chemotherapy, survivors frequently have lifelong health issues from the therapy they received as a child [11]. Despite the outcome of children with neuroblastoma has improved over the last three decades, long-term survivors remain at significant risk for a second malignancy such as head and neck cancer [12]. Therefore, targeted and less toxic therapies are urgently required to treat this disease, to improve the quality of life of the survivors and possibly to prevent the occurrence of a second malignancy.

Natural compounds, including tannins, are complex multiple-target molecules, whose activities have been extensively studied, not only in term of neuroprotection, but also as modulators of multiple signal transduction pathways [13, 14]. This broad spectrum of different properties, which strongly depend on their concentration at the target sites, makes them suitable candidates for the treatment of both brain cancer and neurodegenerative disorders. Information about selective cytotoxic effects of hydrolysable tannins on neuroblastoma cells, however, is still limited. The aim of the present work was to assess cytotoxic potential of ENC in human neuroblastoma SH-SY5Y cells, in order to evaluate its potential as an additional strategy for monotherapy or combination treatments for neuroblastoma. ENC-mediated cytotoxicity was studied by assessing cell proliferation inhibition and apoptosis induction, important features for potential anti-cancer properties.

Human neuroblastoma SH-SY5Y cells were treated with increasing concentrations of ENC for 24, 48, and 72 hours. The extract caused a concentration- and time-dependent decrease in cell viability and the IC_{50} value at the selected time points were: 24h 142.8±20.2 µg/mL; 48h, 109.3±16.6 µg/mL; 72h, 82.5±8.6 µg/mL (Figure 1). It is important to outline that the amounts of total separated tannins and phenolic compounds found in ENC represent ~ 1/10 (if expressed as g ellagic acid equivalent, EAE /100 g) or ~ 1/5 (if expresssed as g gallic acid equivalent, GAE/100 g) of the total amount used [6] (see also below in the Experimental section).

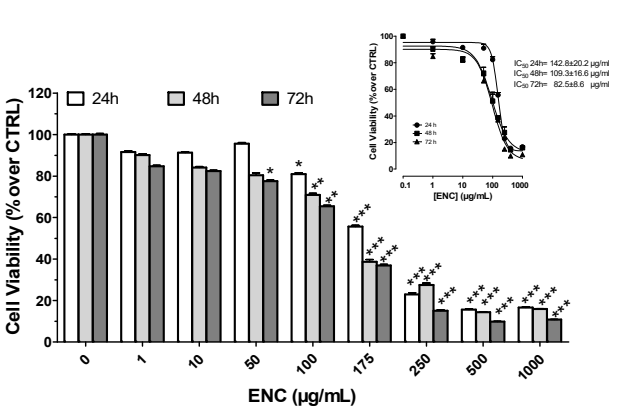

Figure 1: Time- and concentration-dependent cytotoxicity elicited by Natural Extract Chestnut bark (ENC) on human neuroblastoma SH-SY5Y cells. In the ordinate scale, viability is reported as percentage of untreated cells (controls, CTRL). Values are means ± S.E.M. of 5-6 independent experiments in which 4 points/concentration/time were run. Statistical significance was assessed by using ANOVA followed by Dunnett post test. *P<0.05, **P<0.01, ***P<0.001 vs CTRL. Insert: graphical determination of ENC IC_{50} after 24, 48 and 72h of treatment. Values were fitted according to a sigmoid equation with variable slope.

Consequently, it is reasonable to consider that the "effective" total concentration of tannins and phenolic compounds operating in the assay should be 1/10 or 1/5 of that nominally reported. The differences in cell survival detected by MTT assay were mirrored by differences in cell morphology. Consistent changes in the number and size were in fact observed between unexposed cells (controls) and those treated with increasing concentration of the extract for 24h (Figure 2). Treated cells underwent progressively to rounding and granulation compared with unexposed cells. In particular, only some SH-SY5Y cells displayed an irregular body and appeared shrunken at 100 µg/mL (grade 1 of cytotoxicity), while at higher concentrations (250-500 µg/mL), membrane blebbing, ballooning, chromatin condensation, formation of apoptotic bodies, accompanied by a tendency to round-up and detach from the culture plate were evident (grade 2-3 of cytotoxicity), suggesting an apoptotic mechanism for cell death. Interestingly, 24h of treatment with A23187, the calcium ionophore, which induce apoptosis in neurons and often used as positive control, caused a concentration-dependent decrease in cell viability (Figure 1 panel A of supporting materials) paralleled by changes in cell morphology (Figure 1 panel B of supporting materials). The extrapolated IC_{50} was 13.6 µM and the treatment with 10 µM for 24h, which resulted in approximately 50% reduction in SH-SY5Y cells viability, was thus chosen for further experiments.

Following drug treatment, a cell line may or may not be able to restore its activity. In the case of cancer cells, the degree of reversibility of the cytotoxic effects displayed by drugs is of greatest importance. It is in fact imperative to check between reversible and irreversible damage, to prove the existence of the so-called "point of no return", i.e. the limit line between cell injury and cell death. In order to determine if ENC-mediated cytotoxicity was reversible or irreversible, SH-SY5Y cells were treated for 24h with the extract, which was then washed off, and incubated with fresh serum-containing medium for additional 24- or 48-h. As reported in Figure 3, ENC caused an irreversible cytotoxic effect against SH-SY5Y cells at 100 µg/mL, since a drastic decrease in the viability occurred even after incubation with drug-free medium for an extra 48 h period. At 250-1000 µg/mL cell viability, however, already drastically reduced by the treatment, was almost comparable after 24 or 48h of incubation without ENC. An irreversible cytotoxic effects was observed also after the treatment with 10 µM A23187 for 24h (Figure 1 panel C of supporting materials). Flow cytometry

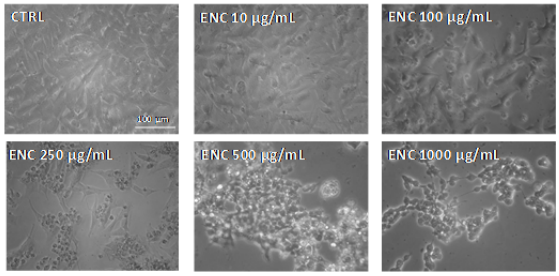

Figure 2: Morphological changes of human neuroblastoma SH-SY5Y cells treated with Natural Extract Chestnut bark (ENC) for 24h. Controls represent untreated cells. Each photograph was representative of three independent observations (scale bar 100 µm OM 100X).

analysis was used to further investigate the mechanisms underlying SH-SY5Y cell death caused by 24h of treatment with ENC. A concentration-dependent rise in the number of sub-G0/G1 hypodiploid cells, which was accompanied by a reduction in the percentage of those in the G0/G1 was observed (Figure 4). The effect was significant at 250 µg/mL (subG0/G1 +11.1%, G0/G1 –22.6%, P<0.01 vs CTRL) and reached the maximum at the highest concentration tested (subG0/G1 +32.5%, G0/G1 –34.8%, P<0.01 vs CTRL).

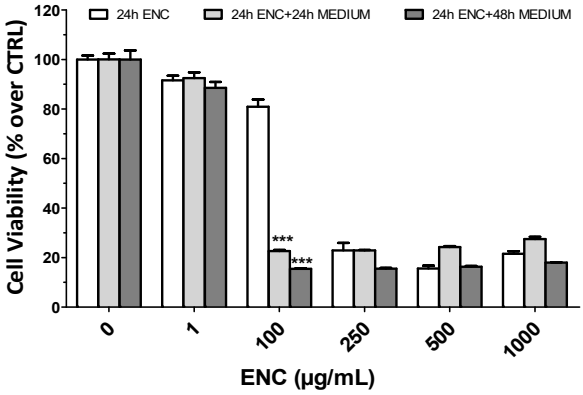

Figure 3: Reversible or irreversible cytotoxic effect caused by Natural Extract Chestnut bark (ENC). SH-SY5Y cells were treated for 24h with ENC (1-1000 µg/mL), which was then washed off, and incubated with fresh serum-containing medium for additional 24 or 48h. After each time point, MTT test was performed. In the ordinate scale, viability is reported as percentage of untreated cells (controls). Values are means ± S.E.M. of 4-5 independent experiments in which 4 points/concentration/time were run. Statistical significance was assessed by using ANOVA followed by Dunnett post test. ***P<0.001 vs 24h ENC-treated cells (white bar).

Also DAPI staining revealed characteristic apoptotic changes like chromatin condensation, nuclear pyknosis, elevated number of nuclear body fragments and irregular edges around the nucleus, which increased in a concentration-dependent manner upon 24h ENC treatment (asterisk in Figure 5). As occurred after ENC treatment, A23187-induced apoptosis of SH-SY5Y cells as highlighted by the significant increase in the subG0/G1 hypodiploid cells (+26.3±3.4%, P<0.001 vs CTRL), the reduction in the percentage of those in the G0/G1 phase (-25.9±2.1%, P<0.001 vs CTRL) (Figure 1, panel D of supporting materials) as well as by the increase in DAPI positive cells (Figure 1, panel E of supporting materials). To explain the mechanism(s) at the basis of these effects, we should consider that the pro-oxidant activity of ENC may be crucial. Polyphenol, in fact, either by themselves or in the presence of copper, can cause oxidative strand breakage in DNA, leading also to base modifications [15-18]. This is mainly due to their structural features, which can form oxygen radicals especially in the presence of transition metal ions [16, 19]. Thus we can hypothesize that ENC-mediated cytotoxicity could depend on pro-oxidant

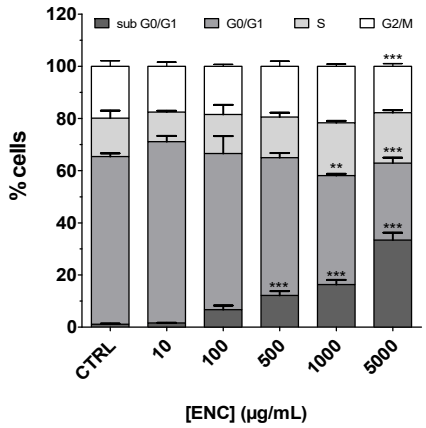

Figure 4: Effects of Natural Extract Chestnut bark (ENC) treatment on SH-SY5Y cell cycle. Percentage of cells in subG0/G1, G0/G1, s and G2/M phase after treatment with increasing concentration of ENC for 24h. Values are means ± S.E.M. of 4-5 independent experiments in which 4 points/concentration/time were run. Statistical significance was assessed by using ANOVA followed by Dunnett post test. ** P<0.01, ***P<0.001 vs untreated cells (CTRL).

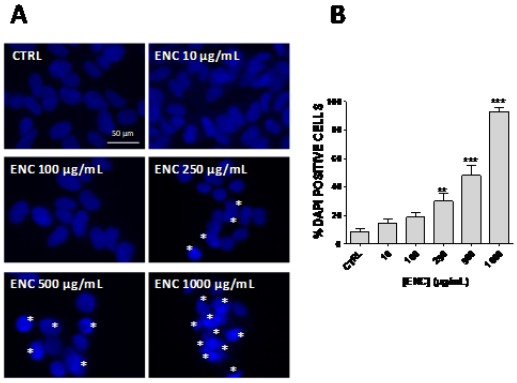

Figure 5: Natural Extract Chestnut bark (ENC)-induced DNA condensation and damage in SH-SY5Y cell as determined by DAPI staining. *Panel A*: Morphological comparison (scale bar 50 µm, OM 400X). Asterisks indicate cells with fragmented nuclei and condensed DNA and considered as apoptotic. Each photograph was representative of three independent observations. **Panel B:** quantitative analysis of untreated (CTRL) and ENC-treated (10-1000 µg/mL for 24h) cells. Values are shown as mean ± S.E.M. of 5 independent experiments. Statistical evaluation was performed by using ANOVA followed by Dunnett *post test*. ** P<0.01, ***P<0.001 vs CTRL.

activity of the extract, as already suggested [9]. This can be of particular importance due also to the difference between cancer and normal cells in terms of redox status, which allows pro-oxidant drugs to selectively kill the former cells by ROS generation [20].

ENC, however, is rich in vescalagin, castalagin, vescalin, castalin, gallic acid and ellagic acid [6]. Data from the literature suggest that 1-10 µM vescalagin possess redox-independent antitumoral activity, owing to a selective inhibition of the alfa isoform topisomerase-mediated decatenation of kDNA [21]. Vescalagin effects are specific and selective, at variance with those of many other polyphenols such as epigallocatechin gallate, which inhibits both isoforms of topoisomerase 2 by a redox-dependent mechanism [22]. Vescalagin can also suppress the EGFR autophosphorylation in human colon carcinoma cells [23], activity shared by castalagin, but not by ellagic acid. In the experiments performed in the present study, when using ENC at amount close to IC_{50} value, the final concentration of vescalagin and castalagin was ~2-5 µM, a value very close to that at which alfa topoisomerase inhibition [21] or EGFR autophosphorylation [23] occurs, further supporting the possibility of a non-redox mechanism at the basis of ENC cytotoxic effects. Interestingly, ENC has been proven to induce extrinsic pathway-mediated apoptosis also in Jurkat cells [24] and preliminary data from our laboratory showed also significant pro-apoptotic activity in the human astrocytoma U-373 MG cell line, an observation which is in agreement with what already reported in the same cells after *Castanea sativa* ethanolic- or methanolic-bark extract challenge [25]. Finally, recent data showed that the treatment of rat cortical slices with ENC up to 200 µg/mL did not elicit tissue cytotoxicity [10], suggesting a potential safe profile of the extract toward healthy tissue, as described also for other natural compounds [26].

In conclusion, despite great progress in the outcome during the last years, the overall survival rate for late stage/high-risk neuroblastoma patients is still critical [12]. This drives the research forward novel drugs or novel adjuvant therapy to be administered with current chemotherapeutic agents, with the final aim to find a treatment with a wide margin of safety towards non-cancer cells. Owing to their pleiotropic properties, natural products have always been an important source of anticancer agents, being also the starting point in the design and development of more than 60% of the clinically used chemotherapy drugs [27]. The present results, which highlighted the apoptotic-inducing activity of ENC, represents the very first step to explore its potential within the frame of neuroblastoma treatment. ENC properties should be further exploited also in term of synergism with established anticancer agents since it might boost cytotoxicity to cancer cells, affect the tumor environment and/or help to mitigate drug-associated toxicity.

Experimental

Materials: The extract obtained by the bark of *Castanea sativa* Mill (ENC®, reported in the text as ENC) was supplied by SilvaTeam (San Michele di Mondovì, Italy). Qualitative and quantitative analysis of ENC composition was performed by HPLC-DAD-MS, as already described [6]. Tannins and phenolic compounds found were 10.7±0.3 g ellagic acid equivalent, EAE/100 g, among which the most representative were vescalagin (2.31±0.05 EAE/100 g), castalagin (2.26±0.07 EAE/100 g), ellagic acid (1.70±0.05 EAE/100 g), gallic acid (1.25±0.04 EAE/100 g), castalin (0.69±0.02 EAE/100 g), vescalin (0.56±0.02 EAE/100 g).

Cell Cultures, ENC treatments and cell viability assay: Human SH-SY5Y neuroblastoma cells were grown in standard conditions, in a humidified atmosphere of 95% air and 5% CO_2 at 37 C [9]. ENC was prepared immediately before use as a stock solution (10 mg/mL in PBS) and pH was adjusted to 7.5 before dilution to the desired final concentration. The solution was sterile filtered by passage through a 0.2-micron sterile filter. To assess ENC effects, SH-SY5Y cells ($8x10^4$ cells/mL, final volume 200 µl) were treated with the extract (0-1000 µg/mL) for 24, 48 or 72 h. For the latter two time points ENC solution was renewed every 24h. At the end of the treatment, MTT assay was performed as already described [28]. Reversibility of the ENC effect was tested by treating cells for 24h with the extract, adding then fresh ENC-free culture medium and assessing the cell viability following 24h or 48h of incubation.

Apoptosis assays: ENC-induced apoptosis was examined by a phase-contrast light microscope and results were quoted according to the grade scale described in USP 28 (United States Pharmacopeia edition 2005) (grades 0-4) for assessment of the cytotoxic potential of tested materials [9, 10] as follows: grade 0 - none reactivity (discrete intracytoplasmic granules, no cell lysis); grade 1 - slight reactivity (no more than 20% of the cells are round, loosely attached and without intracytoplasmic granules; occasional lysed cells are present); grade 2 - mild reactivity (no more than 50 % of the cells are round and devoid of intracytoplasmic granules, no extensive cell lysis and empty areas between cells); grade 3 - moderate (up to 70% of cells are rounded or lysed); grade 4 - severe (nearly complete

destruction of the cells). Cell cycle and sub-G0/G1 population as well as nuclear morphology analysis (DAPI staining) were also performed by using flow cytometry and fluorescence microscopy, respectively [9, 29, 30].

Analysis of Data: Data was collected as quadruplicate from at least 5-6 independent experiments. The results were expressed as means ± S.E.M.. Statistical significance was assessed by using ANOVA followed by Dunnett *post test*). In all comparisons, the level of statistical significance (*P*) was set at 0.05.

Supplementary data: Cytotoxicity elicited by 24h treatment with the positive control A23187 (calcium ionophore) on human neuroblastoma SH-SY5Y cells as MTT assay, morphological changes assessed by contrast phase microscopy, reversible or irreversible cytotoxic effect, effects on cell cycle as well as DAPI staining are also available.

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