

(-)-Epicatechin Enhances Etoposide-induced Antileukaemic Effect in Rats with Acute Myeloid Leukaemia

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Abstract. *Background:* (-)-Epicatechin (EC) is a naturally occurring compound which induces oxidative DNA damage in human acute myeloid leukaemia (AML) cells. *Aim:* The aim of the study was to examine the influence of EC on the antileukaemic effect of etoposide in rats with AML. *Materials and Methods:* Brown Norway rats with AML were treated with EC for 23 days and etoposide was administered for the last three days of the experiment. Bone marrow and splenic cell apoptosis was investigated by flow cytometry using annexin V-allophycocyanin staining. The oxidative status was investigated in homogenates of the liver. *Results:* EC was found to increase the *in vivo* apoptotic effect of etoposide resulting in the decrease of the percentage of leukaemia cells in EC-treated rats in comparison to those treated with etoposide only. Investigation of malondialdehyde and ferric ion-reducing ability of plasma levels indicated that EC increases the oxidative stress induced by etoposide in leukaemic rats. *Conclusion:* EC can enhance the antileukaemia properties of etoposide *in vivo* through augmentation of oxidative stress.

Acute myeloid leukaemia (AML) accounts for 80% of all acute leukaemia cases in adult humans (1). In developed countries, AML occurs with a frequency of 1/100,000, and the number of cases is increasing every year (2). Despite immense steps forward in studies on the pathomechanism of the disease, identification of new prognostic factors and modification of treatment protocols (3, 4), AML remains a

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disease which is difficult to cure. Chemotherapeutic treatment in individuals below 60 years of age usually results in complete remission; however, in the majority of the patients, it eventually leads to recurrence of the disease and death (5). In patients over 60 years of age, the results of the treatment are much worse (5). Therefore, ways to increase therapeutic index of this disease are still being sought.

New compounds which can be used in future targeted AML therapy or complementary treatment to enhance the effect of conventional anticancer drugs are being extensively studied (6, 7). Plant polyphenols are among the compounds which arouse interest in this field. These compounds are known for selective, cytotoxic effect against cancer cells and relatively low toxicity towards normal cells (8, 9). Polyphenols from the catechin group, such as (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) have been shown to be cytotoxic to AML cells (10). These two catechins inhibited the growth and induced apoptosis of human promyelocytic leukaemia HL-60 cells by reducing the levels of B-cell lymphoma 2 (BCL-2) protein and activation of caspase-3 (10). EGCG also induced necrosis-like cell death of chronic myeloid leukaemia K562 cells and in this way it overcome apoptosis resistance (11).

The generation of highly toxic radicals seems to play an important role in the cytotoxic action of EGCG in leukaemia cells derived from myeloid precursors with a high constitutive activity of myeloperoxidase (MPO) (12, 13). Nakazato *et al.* (13) demonstrated that EGCG increases oxidative stress in AML cells and in the HL-60 cell line derived from the human acute promyelocytic leukaemia, using the MPO/H₂O₂/halide system.

Recently, it was demonstrated that catechin hydrate, another component of green tea, exerted a dose-dependent apoptotic effect in breast cancer MCF-7 and cervical carcinoma SiHa cells by inducing expression of proapoptotic genes (14). Most studies on the anticancer effects of

catechins focused on EGCG, the most active component of green tea. However, *in vivo* research on the anticancer activity of (–)-epicatechin (EC), which is characterized as having the best bioavailability among other green tea catechins, is very limited (15).

In our previous studies, EC administered orally at a high dose of 40 mg/kg b.w. (non-cytotoxic) for 23 days, demonstrated antileukaemic activity in a rat model of AML *in vivo*. This polyphenol induces apoptosis of leukaemia cells, leading to a decrease in their number in the spleen of the sick animals (16). However, Oikawa *et al.* (17) proved that EC undergoes MPO-catalyzed one-electron oxidation and induces oxidative DNA damage in HL-60 cells, by the increase of intracellular production of H₂O₂.

One of the cytostatic drugs used in AML treatment is etoposide which, similarly to EC, undergoes one-electron oxidation catalyzed by MPO (18). The mechanism of anticancer activity of the etoposide is based on inhibition of the topoisomerase II-DNA cleavable complex, which leads to the formation of DNA strand breaks and to apoptosis of cancer cells (19). This drug is used in a wide-spectrum treatment of both solid tumors and leukaemias, but the effect of its genotoxicity is especially pronounced in myeloid precursors with high MPO activity (18).

Etoposide phenoxyl radicals formed by MPO may enhance the genotoxic effects of etoposide in these cells (18). In addition, MPO converts etoposide to *O*-demethylated metabolites, such as catechol and quinone, which possess much stronger pro-oxidant activities than etoposide and influence topoisomerase II activity with similar strength as this cytostatic (20, 21).

Taking into account that more free radicals usually arise in cancer cells than in normal cells (22), it can be supposed that EC will enhance etoposide activity in leukaemia cells more than in their normal counterparts.

The aim of this study was to determine whether EC modifies etoposide activity in bone marrow and spleen cells in rats with promyelocytic leukaemia.

Materials and Methods

Chemicals. Reagents including EC, propidium iodide (PI), red blood cell lysing buffer with ammonium chloride, polyethylene glycol, polysorbate 80, benzyl alcohol, citric acid, ethanol and all reagents used in ferric ion reducing ability of plasma (FRAP), malondialdehyde (MDA) and superoxide dismutase (SOD) investigation were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). VEPESID (etoposide) was purchased from Bristol-Myers Squibb S.p.A. (Sermoneta, Italy). RPMI 1640 medium, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from PAA (Pasching, Austria). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45R monoclonal antibody, annexin-V-allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD) were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). (PE)-conjugated goat anti-mouse antibody specific

against μ chain was acquired from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Animals. Male Brown Norway (BN/CrIcmd) rats (297.2±3.6 g) were obtained from the Animal Center, Polish Academy of Sciences Medical Research Center (Warsaw, Poland). The animals were housed in plastic cages and kept under standard conditions of 22°C and 50-60% humidity, with a 12-h light-dark cycle. They were fed a synthetic pellet diet (Morawski Label Feed, Kcynia, Poland) and had free access to water.

BN rat myeloid leukaemia model. BN rats with acute myeloid leukaemia (BNML) are regarded as one of the best animal models of myeloid leukaemia due to their many similarities to human AML, e.g. growth pattern (23). In this experimental model, leukaemia is transplantable and BNML cells are inoculated into immunologically competent rats. In the present study, BNML was developed after intravenous inoculation with 10⁶ spleen-derived leukaemia cells (kindly provided by Professor A.C.M. Martens, Utrecht University, the Netherlands) in PBS. Experiments were performed in accordance with legal requirements, under a licence granted by the Jagiellonian University Ethical Committee.

Treatment protocol. The doses of EC (20 and 40 mg/kg b.w.) were chosen based on our previous research in which EC at a dose of 40 mg/kg b.w. significantly increased apoptosis, while a lower dose (20 mg/kg b.w.) did not induce such an effect on BNML cells of leukaemic rats (16). EC was dissolved in hot water and, after cooling, was administered by oral gavage (40 mg/kg b.w. in 0.5 ml of water) from the second day after the inoculation of BNML cells, every 24 hours until day 23 of leukaemia development. In the study, 50 mg of etoposide/kg b.w. of rat was used, which induced severe DNA damage in the bone marrow of healthy mice (24). Etoposide was administered every 24 hours for three consecutive days starting on the 21st day of leukaemia development. Etoposide was administered intraperitoneally to avoid possible influence of EC on its intestinal bioavailability. Control rats were administered with solvents of investigated compounds. Six to eight leukemic rats were included in each group. Six healthy control and EC-treated rats were also used to investigate the influence of EC on the oxidative status. These rats were killed 1.5 hour after etoposide and 2 hours after EC administration. Turner *et al.* (24) observed the greatest DNA damage in the bone marrow of mice one hour after peritoneal administration of etoposide. Behaviour, weight and general condition of the animals were monitored.

Organ excision and cell isolation. Rats were killed by cervical dislocation under vetbutal anaesthesia. The right femurs were removed and the bone marrow was used for the preparation of smears using a small paintbrush. Bone marrow from left femurs was flushed with 1 ml FBS and then washed once in PBS (230 \times g, 10 min, 4°C). Spleens were excised, weighed and macerated into a Petri dish filled with RPMI-1640 medium, and subsequently centrifuged (230 \times g, 10 min, 4°C). Livers were excised, immediately frozen and stored at –80°C until redox state investigation. In order to examine cells by flow cytometry, the isolated cells were counted in a Bürker haemocytometer and erythrocytes were lysed with red blood cell-lysing buffer containing 0.83% ammonium chloride in 0.01 M Tris buffer (pH 7.5).

Cytological study of bone marrow. Smears were stained using May-Grünwald-Giemsa stain and examined by counting approximately 500 cells/slides on three slides from each animal using an Olympus CX-40 microscope (Olympus, Tokyo, Japan).

Detection of BNML cells and analysis of apoptotic and necrotic cell death. Isolated cells (1×10^6) were incubated for 45 min on ice in 100- μ l PBS containing 5% FBS and 10% mouse primary monoclonal anti-RM124 antibody (kindly provided by Professor A.C.M. Martens), which recognises the specific epitope of the leukaemia cells (23). After incubation, the cells were washed in PBS (230 $\times g$, 10 min, 4°C). Next, they were incubated with secondary goat anti-mouse monoclonal antibody conjugated to R-PE for 30 min and washed in PBS (230 $\times g$, 10 min, 4°C). The cells were then incubated with FITC-conjugated mouse anti-rat CD45R monoclonal antibody for 20 min to evaluate the nonspecific binding of the secondary antibody to B-lymphocytes. Subsequently, the cells were stained with annexin V-APC (An-APC) and 7AAD according to the instructions provided by the manufacturer and incubated in the dark for 15 min. All the steps were performed on ice. The cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Apoptotic cells were defined as APC-An-positive and 7AAD-negative, while necrotic and/or late apoptotic cells were APC-An- and 7AAD-positive, necrotic cells were 7AAD-positive and APC-An-negative. Results were expressed as the percentage of apoptotic or necrotic cells.

Measurement of FRAP. The method of Benzie and Strain (25) was used to determine FRAP as an indicator of the antioxidant content. FRAP values were obtained by measuring ferric to ferrous ion reduction at low pH (300 mmol acetate buffer, pH 3.6), coupled with tripyridyltriazine, a blue-colored complex, whose absorbance was measured at 593 nm. Tissues were homogenized in 0.05 M phosphate buffer (pH 7.8) to 10% final concentration. Homogenized tissues were centrifuged at 1000 $\times g$ for 15 min (0–4°C). The supernatant was drawn and the pellet was discarded. Each sample (0.1 ml) was mixed with 3 ml reagent mixture [30 ml, consisting of 25 ml of acetate buffer (300 mM; pH 3.6), 2.5 ml 2,4,6-Tris(2-pyridyl)-*S*-triazine (5 mM TPTZ) in 40 mM HCl, and 2.5 ml ferric solution (20 mM FeCl₃) in distilled water]. The FRAP was calculated by preparing a standard curve between 0 and 1 mM of known Fe²⁺ sample using FeSO₄·7H₂O as the standard. The blank contained FRAP reagent mixture. Reactions were performed for 5 min at 37°C and analyzed spectrophotometrically. FRAP values are expressed as μ mol/mg of protein.

Investigation of lipid peroxidation. The concentration of MDA, a product of lipid peroxidation, was measured in liver homogenates according to the method of Buege and Aust (26), based on thiobarbituric acid (TBA) reactivity. Reaction of MDA with TBA followed by measurement of the pink chromophore product was used for quantitative determination of MDA. Each sample (0.5 ml) was mixed with 1.5 ml reagent mixture (TBA/TCA/HCl reagent by diluting the stock solution 4-fold in water) to maintain a reagent/sample ratio of 2:1 (v/v). Working solution was prepared by diluting the stock solution of TBA/TCA/HCl 4-fold in water. Butylated hydroxytoluene (BHT) in ethanol was added to a final concentration of 0.03%. After shaking the tube, the mixture was placed into boiling water bath for 15 min. After cooling in cold

water, the obtained chromogen was extracted with 3.0-ml *n*-butyl alcohol by vigorous shaking for 1 min. Separation of the organic phase was facilitated by centrifugation at 1000 $\times g$ for 10 min. The TBA-MDA adduct was quantified using fluorescence emission at 553 nm upon assessment of cell toxicity excitation at 532 nm. The MDA concentration was measured by preparing a standard curve between 0 and 50 nmol MDA/sample using 1,1,3,3-tetramethoxypropane as the standard. MDA concentrations are expressed as nmol/mg of protein. The measurements were performed by using a spectrofluorometer (Perkin Elmer LS-5, USA).

Measurements of SOD activity. SOD activity was investigated by the method of Spitz and Oberley (27) in homogenates from liver. Xanthine-xanthine oxidase was utilized to generate a superoxide flux. Nitro-blue tetrazolium (NBT) reduction by O₂⁻ to blue formazan was followed at 560 nm in a Marcel Media (Poland) spectrophotometer at room temperature. The rate of NBT reduction in the absence of tissue was used as the reference rate (0.020 \pm 0.005 AU/min). When increasing amounts of protein (containing SOD activity) were added to the system, the NBT reduction rate was progressively inhibited. The amount of inhibition was defined as a percentage of the reference rate of NBT reduction, when SOD activity was absent. The data were plotted as the percentage inhibition vs. protein concentration. One unit of activity was defined as the amount of protein necessary to reduce the reference rate to 50% of maximum inhibition. The assay mixture contained catalase to remove H₂O₂ and diethylenetriaminepentaacetic acid (DETAPAC) to chelate metal ions capable of redox cycling and interference with the assay system. The measurements were performed spectrophotometrically using a Marcel Media spectrophotometer (Poland).

Tissues were homogenized in 0.05 M phosphate buffer (pH 7.8) to a final concentration of 10%. Homogenates were sonicated and then centrifuged at 1000 $\times g$ for 15 min (0–4°C). The supernatant was drawn and the pellet was discarded. Each supernatant (0.1 ml) was mixed with 0.8 ml reagent mixture containing: bovine serum albumin (BSA) in DETAPAC buffer, 50 mM potassium phosphate monobasic and dibasic buffer, (pH 7.8); 1 mM DETAPAC; 0.13 mg/ml BSA (12.90 ml); 1.0 U bovine liver catalase (0.5 ml); 5.6 $\times 10^{-5}$ M NBT (0.5 ml); 0.1 mM xanthine (1.7 ml); 0.05 M potassium phosphate buffer (0.3 ml); 50.0 μ M bathocuproine disulfonate disodium salt (BCS) (0.1 ml) and 0.1 ml of the xanthine oxidase, 13.2 U/ml to achieve the required reference rate. All data were expressed in units of SOD activity per milligram of protein.

Statistical analysis. One-way analysis of variance ANOVA and post-hoc Tukey test were used to calculate the statistical significance between groups. Values of SOD activity were assessed by the nonparametric Mann-Whitney test. Data are represented as the mean \pm standard error of the mean.

Results

Splenic weight. The weight of spleen from leukaemic rats was increased several fold due to the colonization of leukaemia cells in this organ in comparison to the healthy animals. Significant decrease in splenic weight of the rats was observed when treated with EC at the dose of 40 mg/kg b.w. both with and without etoposide in comparison to the control (Figure 1). EC, used at the dose of 20 mg/kg b.w. did

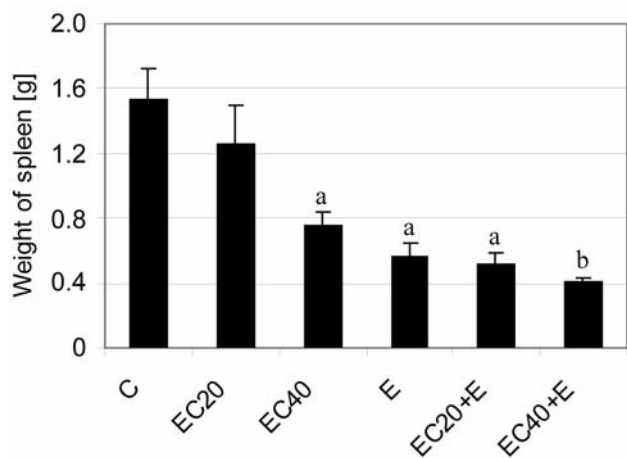


Figure 1. Splenic weight of the BNML rats treated with (-)-epicatechin (EC) and/or etoposide. C, control rats; EC20, rats treated with EC (20 mg/kg b.w.); EC40, rats treated with EC (40 mg/kg b.w.); E, rats treated with etoposide (50 mg/kg b.w.); EC20+E, rats treated with EC (20 mg/kg b.w.) and etoposide (50 mg/kg b.w.); EC40+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as mean±standard error of the mean. ^a*p*<0.05 vs. C; ^b*p*<0.001 vs. C.

not influence the splenic weight (Figure 1). Pre-treatment with EC (40 mg/kg b.w.) followed by co-administration with etoposide led to a non significant decrease of splenic weight in comparison to etoposide administration alone. The lower dose of EC did not influence the spleen weight, changed by etoposide (Figure 1).

Cytological evaluation of bone marrow smears. BNML leukaemia is characterized by slow growth and a strong suppression of haematopoiesis (23). Cytological studies of bone marrow were used to compare the effects of EC and etoposide on the percentage of nucleated erythroid cells, lymphocytes, monocytes/macrophages and granulocytic cells.

Promyelocytes accounted for about 40% of all nucleated bone marrow cells in the BNML rats, and the percentages of all normal haematopoietic cells of BNML rats were reduced in their bone marrow in comparison to the healthy controls (Table I).

EC administered to the BNML rats at a dose of 20 mg/kg b.w. did not significantly influence the percentages of bone marrow cells, nor did it influence the effect of etoposide in these cells (Table I). The BNML rats receiving EC at a dose of 40 mg/kg b.w. exhibited a significant decrease in the number of promyelocytes by about 62% and increase in the percentages of normal nucleated erythroid cells, lymphocytes and macrophages, in comparison to the control group (Table I). Therefore, the dose of 40 mg/kg b.w. of EC was used for the subsequent experiments.

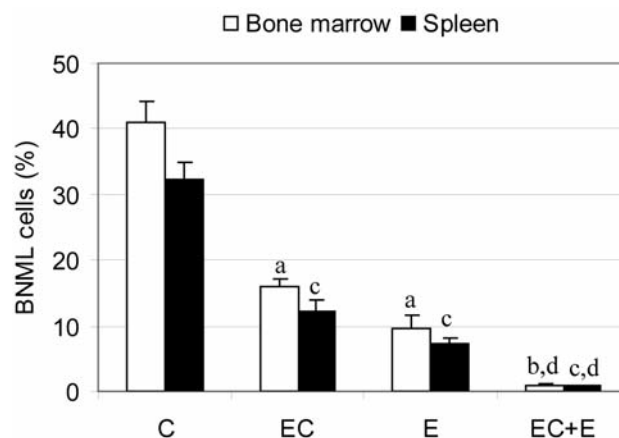


Figure 2. The level of leukaemia cells in the spleen and bone marrow. C, Control rats; EC, rats treated with (-)-epicatechin (EC) (40 mg/kg b.w.); E, rats treated with etoposide (50 mg/kg b.w.); EC+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean±standard error of the mean. ^a*p*<0.05 vs. C; ^b*p*<0.01 vs. C; ^c*p*<0.05 vs. C; ^d*p*<0.05 vs. E and EC.

Etoposide administration caused a decrease in cellularity of the bone marrow. A decrease in the percentage of promyelocytes by 81% in comparison to the control group was observed (Table I).

Co-administration of EC (40 mg/kg b.w.) and etoposide resulted in further decrease of ~92% in the number of promyelocytes of total nucleated cells, as compared to the control, and by 59% compared to etoposide-treated group. Additionally, EC prevented decrease in the number of normal nucleated erythroid cells, lymphocytes, granulocytes and precursors of granulocytes under the influence of etoposide (Table I).

Influence of the treatment with EC/etoposide on apoptosis and the percentage of leukaemia cells. Some polyphenols may enhance antitumor activity of many cytostatics, including etoposide. Taking into account that EC reveals proapoptotic activity in leukaemia cells of BNML rats, as shown in our earlier studies (16), the goal of this study was to compare *in vivo* proapoptotic activity of etoposide vs. EC plus etoposide.

EC reduced the percentage of BNML cells in the spleen and bone marrow in comparison to the untreated control. A similar effect was exerted by etoposide. EC significantly enhanced the effect of etoposide in both examined organs compared to the group treated with cytostatic only (Figure 2).

EC, and etoposide administration increased significantly the percentage of annexin-V positive BNML cells both in the spleen and the bone marrow in comparison to the control (Figure 3A). EC, when administered both before and with

Table I. Cytology of bone marrow isolated from healthy control and leukaemic rats. H, Healthy control rats; C, control leukaemic rats, EC20, rats treated with EC (20 mg/kg b.w.); EC40, rats treated with EC (40 mg/kg b.w.); E, leukaemic rats treated with etoposide (50 mg/kg b.w.); EC20+E, rats treated with EC (20 mg/kg b.w.) and etoposide (50 mg/kg b.w.); EC40+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean percentage of each cell population \pm standard error of the mean. Means with different letters in the same column differ significantly, $p < 0.05$.

Group of rats	Promyelocytes (%)	Granulocytic cells (%)	Nucleated erythroid cells (%)	Lymphocytes (%)	Monocytes/macrophages (%)
H	2.0 \pm 0.1 ^a	34.3 \pm 1.3 ^a	27.6 \pm 1.6 ^a	17.0 \pm 1.0 ^a	1.9 \pm 0.4 ^a
C	37.4 \pm 2.2 ^b	67.7 \pm 5.2 ^b	10.7 \pm 1.3 ^b	5.8 \pm 0.6 ^{b,c}	1.1 \pm 0.1 ^a
EC20	31.6 \pm 5.8 ^b	63.7 \pm 6.6 ^b	13.1 \pm 2.1 ^b	6.3 \pm 2.1 ^{b,c}	0.8 \pm 0.5 ^a
EC40	14.0 \pm 0.5 ^c	48.8 \pm 2.6 ^c	20.7 \pm 3.7 ^a	12.0 \pm 0.1 ^a	2.0 \pm 0.0 ^a
E	6.8 \pm 0.3 ^d	12.3 \pm 2.1 ^d	3.1 \pm 0.3 ^c	4.9 \pm 1.3 ^b	2.1 \pm 0.3 ^a
EC20+E	7.2 \pm 0.8 ^d	13.8 \pm 1.3 ^d	4.2 \pm 2.1 ^{c,d}	3.9 \pm 1.8 ^b	1.6 \pm 0.9 ^a
EC40+E	2.6 \pm 0.1 ^a	17.2 \pm 3.7 ^d	8.8 \pm 1.4 ^{b,d}	9.6 \pm 0.7 ^{a,c}	5.6 \pm 1.5 ^b

etoposide significantly enhanced the percentage of annexin V-positive leukaemia cells, both in the spleen and the bone marrow, in comparison to the treatment with etoposide only (Figure 3A and B). EC and etoposide also induced necrosis of leukaemia cells in the bone marrow of the BNML rats; simultaneous administration of both compounds did not lead to significant intensification of the process (Figure 3B).

FRAP. Owing to the fact that EC and etoposide can induce oxidative damage in myeloid leukaemia cells (17, 18), it can be assumed that they can affect the antioxidant capacity in BNML rats. In order to determine the effect of these compounds on the level of low molecular weight antioxidants, FRAP analysis was performed.

EC did not exert a significant effect on the FRAP level in the liver of healthy rats (110 \pm 22 μ mol/mg protein) in comparison to the control (108 \pm 25 μ mol/mg protein). However, a significant decrease in FRAP values was observed under the influence of EC, and etoposide used separately in rats with leukaemia compared to the control. The highest significant decrease in FRAP values were observed in the group of rats that received both EC and etoposide in comparison to the other groups (Figure 4), indicating that EC enhances the reduction in defence of the tissue by the low molecular weight antioxidants caused by etoposide in the BNML rats.

MDA concentration. Taking into account that apoptosis is accompanied by an increase in lipid peroxidation, in the next step, the extent of lipid peroxidation product, MDA, was assessed.

EC significantly increased MDA levels in BNML rat liver when compared to the control (Figure 5) but did not induce such an effect in healthy rats. The level of MDA in healthy control and healthy EC-treated rats was 0.091 \pm 0.01 and 0.095 \pm 0.0 nmol/mg protein, respectively.

MDA concentration was significantly increased under the influence of etoposide in comparison to the control. Administration of EC before and with etoposide led to a significant increase in the MDA level in comparison to etoposide only (Figure 5), indicating that EC is synergistic with etoposide in the induction of oxidative stress in the BNML rats.

Investigation of SOD activity. SOD is the only defence of the cells responsible for dismutation of O₂^{•-}, therefore disturbances in its function can favour overproduction of reactive oxygen species (ROS).

SOD activity was significantly reduced in the EC-treated BNML rats in comparison to the control (Figure 6). Etoposide treatment induced a significant increase in the activity of SOD when compared to the control. When EC was administered before and with etoposide, a significant decrease in the SOD activity was observed in comparison to the rats treated with etoposide only (Figure 6).

Discussion

Studies by Nakazato *et al.* (13) have shed light on the potential new therapeutic strategy of AML treatment by using ROS-generating agents, such as polyphenol members, the catechins. These authors have demonstrated that EGCG intensified the proapoptotic activity of a low concentration of As₂O₃ in HL-60 cells by MPO-mediated production of highly toxic hydroxyl radical. In this article, we present the first results of studies demonstrating that EC increases the apoptotic action of etoposide in rat myeloid leukaemia cells, leading to a significant decrease in the number of these cells.

EC administered from the initial to late stage of the disease restricted the development of leukaemia. Such an observation resembles the situation when leukaemia recurs after remission. These results are in line with our previous

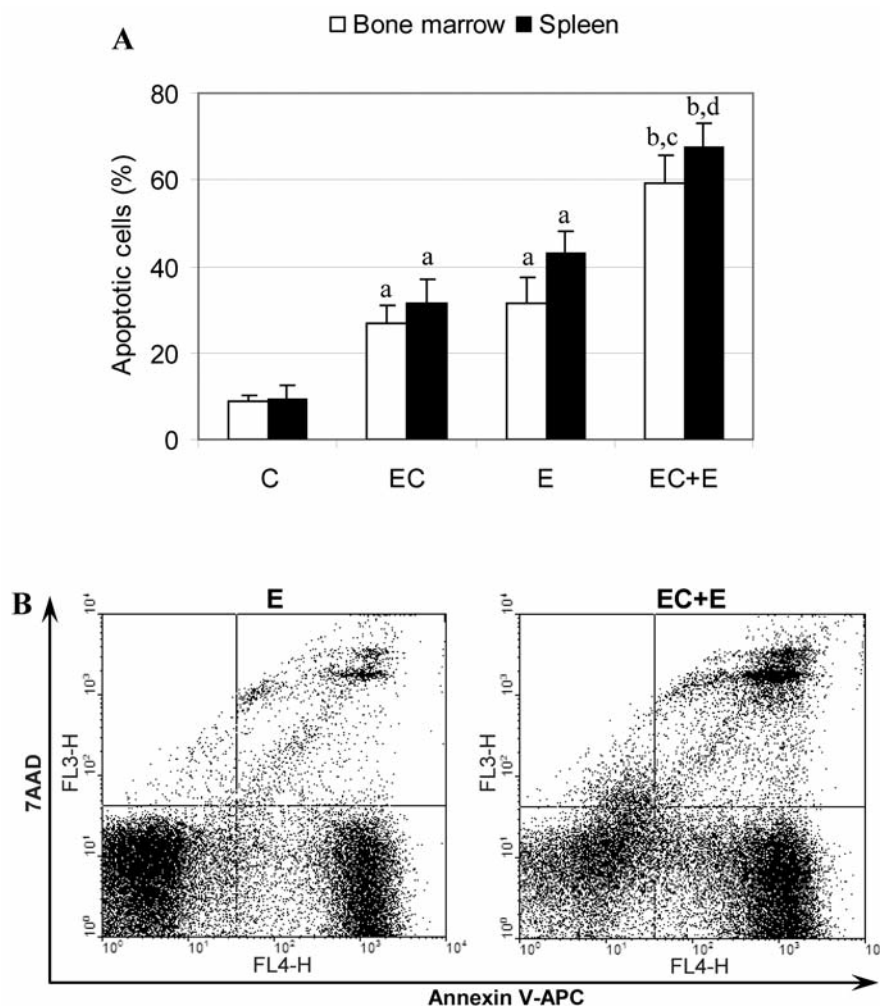


Figure 3. Apoptosis of leukaemia cells isolated from the BNML rats pre-treated with (-)epicatechin (EC) followed by coadministration of etoposide. A: Percentage of annexin V-positive cells. B: Representative dot-plots of annexin V-APC (FL4) vs. 7-AAD (FL3) staining. The cells were isolated from the spleen of leukaemic rats. C, Leukaemia cells isolated from the control rats; EC, leukaemic cells of rats treated with EC (40 mg/kg b.w.); E, leukaemic cells of rats treated with etoposide (50 mg/kg b.w.), leukaemic cells of rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean±standard error of the mean. ^a*p*<0.05 vs. C; ^b*p*<0.001 vs. C; ^c*p*<0.01 vs. EC, and E; ^d*p*<0.01 vs. E.

research which demonstrated the proapoptotic effect of EC in the same model of rat leukaemia leading to a decrease in the number of BNML cells (16).

Cytological evaluation of bone marrow smears proved that EC leads to a decrease in the proportion of promyelocytes, with a simultaneous increase of normal bone marrow cells. A similarly selective activity was shown by EGCG, which induced apoptosis of a multiple myeloma cell line, without such an effect on normal activated mononuclear peripheral blood cells (8).

It can be supposed that one of the proapoptotic mechanisms of EC action may be escalating oxidative stress in leukaemic rats, as evidenced by the significant increase in MDA level in the liver, one of the target organs for BNML

cells. Other studies have demonstrated that oxidative stress-induced apoptosis is accompanied by an increase in lipid oxidation (28). MDA is a product of membrane fatty acid peroxidation formed under the influence of free radicals (29). Peroxidation of mitochondrial membrane lipids leads to the initiation of apoptosis by the release of cytochrome *c* from the mitochondrial inner membrane to the cytosol. (28). However, oxidation of cellular membrane phospholipids may in turn contribute to the loss of plasma membrane asymmetry and externalization of phosphatidylserine (30).

FRAP decrease under the influence EC indicates a reduction in defence of tissue against uncontrolled oxidative reactions, which in turn results in increased MDA levels. The observed decrease in SOD activity in the liver could be due

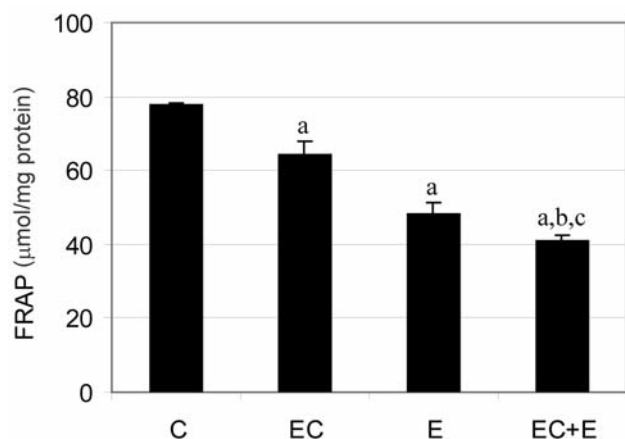


Figure 4. Ferric ion reducing ability of plasma (FRAP) values in the liver of leukaemic rats. C, Control rats; EC, rats treated with (-)-epicatechin (EC) (40 mg/kg b.w.); E, rats treated with etoposide (50 mg/kg b.w.); EC+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean±standard error of the mean. ^a $p < 0.001$ vs. C; ^b $p < 0.001$ vs. EC; ^c $p < 0.05$ vs. E.

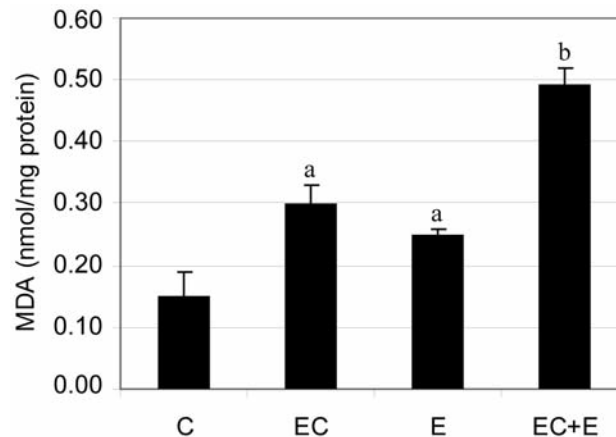


Figure 5. The level of malondialdehyde (MDA) in the liver of leukaemic rats. C, Control rats; EC, rats treated with (-)-epicatechin (EC) (40 mg/kg b.w.); E, rats treated with etoposide (50 mg/kg b.w.); EC+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean±standard error of the mean. ^a $p < 0.01$ vs. C, ^b $p < 0.001$ vs. C, EC, and E.

to the oxidative damage caused by long-term administration of a high dose of EC to leukaemic rats.

It should be added that EC did not significantly affect the MDA levels and FRAP value of healthy rats. Various previous studies have shown that the pro-oxidative effect of polyphenols may occur under conditions of increased oxidative stress, which is found in cancer cells (9, 31). Among examples of polyphenols which have selective antitumor activity in cancer cells and have no such action in normal cells are EGCG and cyanidine 3-rutinozide (9, 31). Cancer cells are sensitive to pro-oxidative agents that may induce DNA damage and activate transcription factors sensitive to stress and which are responsible for cell death (22).

In our earlier studies, we showed that the BNML rats have an increased level of oxidative stress in comparison to healthy animals of the same strain. FRAP values and glutathione level were significantly reduced; however, expression of heme oxygenase-1, an enzyme induced by oxidative stress, and MDA concentrations were significantly higher in the spleen and brain of BNML rats in comparison to the healthy controls (32). Additionally, studies of DNA damage by comet assay showed that the BNML rats also demonstrated elevated oxidative DNA damage compared to healthy rats, which correlated with the proportion of leukaemia cells in these rats (16). Owing to the fact that BNML leukaemia was first induced with dimethylbenzoanthracene, it can be supposed that ROS generated by this carcinogen (33) could initiate the formation of oxidative damage in the BNML cells.

EC as a substrate for MPO may be oxidized to phenoxyl radicals inducing oxidative stress in the leukaemia cells of the myeloid lineage. The studies by Galati *et al.* (34) have

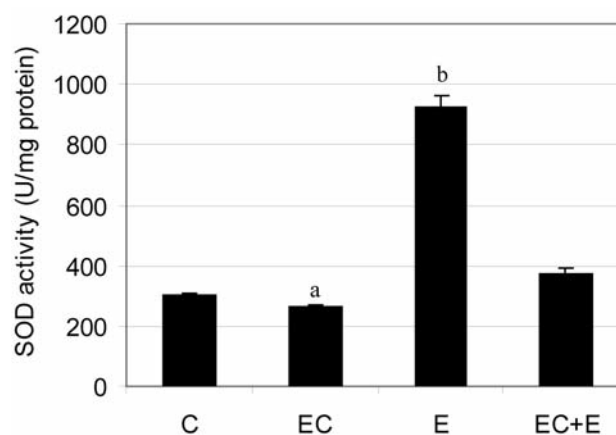


Figure 6. Superoxide dismutase (SOD) activity in the liver of leukaemic rats. C, Control rats; EC, rats treated with (-)-epicatechin (EC) (40 mg/kg b.w.); E, rats treated with etoposide (50 mg/kg b.w.); EC+E, rats treated with EC (40 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean±standard error of the mean. ^a $p < 0.05$ vs. C and EC+E, ^b $p < 0.001$ vs. C, EC, and EC+E.

shown that polyphenol-derived pro-oxidative phenoxyl radicals formed under the influence of MPO may oxidize lipoproteins, lipids, glutathione and beta-NADH, leading to the formation of superoxide radical anion.

Etoposide induced oxidative stress in the BNML rat liver, as evidenced by a decrease in FRAP values and an increase in MDA concentration. A significant increase in SOD activity was observed after the etoposide administration, which may indicate an increase in defence mechanisms

against excessive production of superoxide anion in the tissues. Pro-oxidative activity of etoposide correlated with apoptosis of BNML cells in etoposide-treated rats.

Detection of phosphatidylserine externalization supported the fact that EC enhanced the proapoptotic effect of etoposide in both the investigated organs of leukaemic rats. It is noteworthy that another catechin, EGCG, sensitized doxorubicin-resistant leukaemia cells to apoptosis in tumours of a human carcinoma xenograft model (35). Enhancement of anticancer action of the etoposide was also observed under the action of the flavone wogonin, which potentiated the proapoptotic effect of etoposide in human myeloid leukaemia HL-60 cells and protected normal rat thymocytes from the cytotoxic effect of the drug (36).

Our observations also demonstrate that EC administered before and with etoposide protected rats against the drastic decline in normal nucleated erythroid cells and lymphocytes caused by etoposide. The observed effect is probably due to the fact that the rats which received EC before etoposide administration had a higher pool of normal bone marrow cells in comparison to the animals treated with etoposide only. It is worth mentioning that the study of Takano *et al.* (37) demonstrated the intensification of granulocyte recovery under the influence of (+)-catechin in rats treated with 5-fluorouracil, however, the mechanism of this action has not yet been clarified.

Co-treatment with EC and etoposide caused an increase in oxidative stress, as shown by a significant increase in MDA levels and a decrease in FRAP values compared to EC and etoposide used separately. The reason for the low activity of SOD in the rats treated with both compounds in comparison to those treated with etoposide only may be disturbances in the function of this enzyme under the influence of oxidative damage induced by EC. Elevated levels of oxidative stress correlated with apoptosis of BNML cells in the rats co-treated with EC and etoposide.

The results of this study indicate that EC can modify etoposide activity, contributing to enhancement of its anticancer activity in BNML rats. An increase of oxidative stress by EC appears to be one of the main mechanisms of its synergistic action with etoposide. The administration of EC before and with etoposide partially protects against haemotoxicity caused by etoposide. Therefore, future investigation of its mechanism of action could be useful for enhancing the proapoptotic activity of etoposide towards leukaemia cells while protecting their normal counterparts.

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References

- Sandler DP and Ross JA: Epidemiology of acute leukemia in children and adults. *Sem Oncol* 24: 3-16, 1997.
- Siegel R, Naishadham D and Jemal A: Cancer statistics, 2012. *CA Cancer J Clin* 62: 10-29, 2012.
- Ohno R, Asou N and Ohnishi K: Treatment of acute promyelocytic leukemia. Strategy toward further increase of cure rate. *Leukemia* 17: 1454-1463, 2003.
- Ravandi F, Burnett AK, Agura ED and Kantarjian HM: Progress in the treatment of acute myeloid leukemia. *Cancer* 110: 1900-1910, 2007.
- Jemal A, Tiwari R, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ and Thun MJ: Cancer statistics. *CA Cancer J Clin* 54: 8-29, 2004.
- King ME and Rowe JM: Recent developments in acute myelogenous leukemia therapy. *Oncologist* 12: 14-21, 2007.
- Robak T and Wierzbowska A: Current and emerging therapies for acute myeloid leukemia. *Clin Ter* 31: 2349-2370, 2009.
- Shammas MA, Neri P, Koley H, Batchu RB, Bertheau RC, Munshi V, Prabhala R, Fulciniti MF, Tai YT, Treon SP, Goyal RK, Anderson KC and Munshi NC: Specific killing of multiple myeloma cells by (-)-epigallocatechin-3-gallate extracted from green tea: biologic activity and therapeutic implications. *Blood* 108: 2804-2810, 2006.
- Feng R, Ni HM, Wang SY, Tourkova IL, Shurin MR and Harada H: Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemia cells by induction of oxidative stress. *J Biol Chem* 282: 13468-13476, 2007.
- Han DH and Kim JH: Difference in growth suppression and apoptosis induction of EGCG and EGC on human promyelocytic leukemia HL-60 cells. *Arch Pharm Res* 32: 543-547, 2009.
- Hazawa M, Takahashi K, Sugata S and Kashiwakura I: (-)-Epigallocatechin-3-O-gallate induces nonapoptotic cell death in leukemia cells independent of the 67 kDa laminin receptor. *J Nat Prod* 74: 695-700, 2011.
- Furukawa A, Oikawa S, Murata M, Hiraku Y and Kawanishi S: (-)-Epigallocatechin gallate causes oxidative damage to isolated and cellular DNA. *Biochem Pharmacol* 66: 1769-1778, 2003.
- Nakazato T, Ito K, Miyakawa Y, Kinjo K, Yamada T, Hozumi N, Ikeda Y and Kizaki M: Catechin, a green tea component, rapidly induces apoptosis of myeloid leukemic cells *via* modulation of reactive oxygen species production *in vitro* and inhibits tumor growth *in vivo*. *Haematologica* 90: 317-325, 2005.
- Al-Hazzani AA and Alshatwi AA: Catechin hydrate inhibits proliferation and mediates apoptosis of SiHa human cervical cancer cells. *Food Chem Toxicol* 49: 3281-3286, 2011.
- Chen L, Lee MJ, Li H and Yang CS: Absorption, distribution, elimination of tea polyphenols in rats. *Drug Metab Dispos* 9: 1045-1050, 1997.
- Papież MA, Baran J, Bukowska-Straková K and Wiczkowski W: Antileukemic action of (-)-epicatechin in the spleen of rats with acute myeloid leukemia. *Food Chem Toxicol* 48: 3391-3397, 2010.
- Oikawa S, Furukawa A, Asada H, Hirakawa K and Kawanishi S: Catechins induce oxidative damage to cellular and isolated DNA through the generation of reactive oxygen species. *Free Radic Res* 37: 881-890, 2003.
- Kagan VE, Yalowich JC, Borisenko GG, Tyurina YY, Tyurin VA, Thampatty P and Fabisiak JP: Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. *Mol Pharmacol* 56: 494-506, 1999.

- 19 Berger NA, Chatterjee S, Schmotzer JA and Helms SR: Etoposide (VP-16-213)-induced gene alterations: potential contribution to cell death. *Proc Natl Acad Sci USA* 88: 8740-8743, 1991.
- 20 Haim N, Nemeč J, Roman J and Sinha B: Peroxidase-catalyzed metabolism of etoposide (VP-16–213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res* 47: 5835-5840, 1987.
- 21 Lovett BD, Strumberg D, Blair IA, Pang S and Burden DA: Etoposide metabolites enhance DNA topoisomerase II cleavage near leukemia-associated MLL translocation breakpoints. *Biochemistry* 40: 1159-1170, 2001.
- 22 Schumacker PT: Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* 10: 175-176, 2006.
- 23 Martens AC, Van Bekkum DW and Hagenbeek A: The BN acute myelocytic leukemia (BNML) (a rat model for studying human acute myelocytic leukemia (AML)). *Leukemia* 4: 241-257, 1990.
- 24 Turner SD, Wijnhoven SWP, Tinwell H, Lashford LS, Rafferty JA, Ashby J, Vrieling H and Fairbairn LJ: Assays to predict the genotoxicity of the chromosomal mutagen etoposide – focusing on the best assay. *Mutation Res* 493: 139-147, 2001.
- 25 Benzie I and Strain J: The ferric-reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Anal Biochem* 23: 270-276, 1996.
- 26 Buege JA and Aust SD: Microsomal lipid peroxidation. *Method Enzymol* 53: 302-310, 1978.
- 27 Spitz DR and Oberley LW: An assay for superoxide dismutase activity in mammalian tissue homogenates. *Anal Biochem* 15: 8-18, 1989.
- 28 Ma Y, Ogino T, Kawabata T, Li J, Eguchi K and Okada S: Cupric nitrilotriacetate-induced apoptosis in HL-60 cells: association with lipid peroxidation, release of cytochrome *c* from mitochondria, and activation of caspase-3. *Free Radic Biol Med* 27: 227-233, 1999.
- 29 Benderitter M, Vincent-Genod L, Pouget JP and Voisin P: The cell membrane as a biosensor of oxidative stress induced by radiation exposure: a multiparameter investigation. *Radiat Res* 159: 471-483, 2003.
- 30 Anazetti MC, Melo PS, Durán N and Haun M: Dehydrocrotonin and its derivative, dimethylamide-crotonin induce apoptosis with lipid peroxidation and activation of caspases-2, -6 and -9 in human leukemic cells HL60. *Toxicology* 203: 123-137, 2004.
- 31 Azam S, Hadi N, Khan NU and Had SM: Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro* 18: 555-561, 2004.
- 32 Papież MA, Dybala M, Sowa-Kucma M, Krzysciak W, Taha H, Jozkowicz A and Nowak G: Evaluation of oxidative status and depression-like responses in Brown Norway rats with acute myeloid leukemia. *Prog Neuropsychopharmacol Biol Psychiatry* 33: 596-604, 2009.
- 33 Dimitrova-Shumkovska J, Veenman L, Ristoski T, Leschiner S and Gavish M: Decreases in binding capacity of the mitochondrial 18 kDa translocator protein accompany oxidative stress and pathological signs in rat liver after DMBA exposure. *Toxicol Pathol* 38: 957-968, 2010.
- 34 Galati G, Sabzevari O, Wilson JX and O'Brien PJ: Pro-oxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177: 91-104, 2002.
- 35 Zhang Q, Wei D and Liu J: *In vivo* reversal of doxorubicin resistance by (–)-epigallocatechin gallate in a solid human carcinoma xenograft. *Cancer Lett* 208: 179-186, 2004.
- 36 Lee E, Enomoto R, Suzuki Ch, Ohno M, Ohashi T, Miyauchi A, Tanimoto E, Maeda K, Hirano H, Yokoi T and Sugahara Ch: Wogonin, a plant flavone, potentiates etoposide-induced apoptosis in cancer cells. *Ann NY Acad Sci* 1095: 521-526, 2007.
- 37 Takano F, Tanaka T, Aoi J, Yahagi N and Fushiya S: Protective effect of (+)-catechin against 5-fluorouracil-induced myelosuppression in mice. *Toxicology* 201: 133-142, 2004.

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