DOI: 10.2429/proc.2018.12(2)032 2018;12(2)

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NATURAL XENOBIOTICS INDUCING APOPTOSIS IN HEPATOMA CELLS: IN VITRO STUDY

NATURALNE KSENOBIOTYKI INDUKUJĄCE APOPTOZĘ W KOMÓRKACH NOWOTWORU ZŁOŚLIWEGO WĄTROBY - BADANIA IN VITRO

Abstract: The term "xenobiotics" refers to substances which are not produced within the human body, but may occur in human environment. Some of biologically active chemicals may be synthesized by plants. Many studies have been conducted with regard to plant secondary metabolites and the beneficial impact of various plant-derived compounds on human health has been demonstrated. Recent research have shown chemopreventive properties of catechins. (+)-catechin ((2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol) and (-)-epigallocatechin gallate (EGCG; [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate) are compounds abundant in daily human nutrition. Growing body of evidence reports pro-apoptotic effects of catechins. Both catechins have been shown to modulate intracellular pathways network in tumor cells thereby regulating signaling cascades, which results in programmed death of cancer cells, apoptosis. Apoptosis is one of the most potent mechanisms to defense against cancer. During apoptosis, the activation of caspases results in biochemical and morphological changes associated with specific changes in cell phenotype. Besides apoptosis, tumor cells may be also eliminated from organism *via* necrosis, however the latter process may evoke local inflammation, which in turn may result in detrimental effects within the body. Xenobiotics with potential to cause apoptosis of cancer cells may be considered in modern combined anticancer therapies and/or to assist existing treatments.

Keywords: xenobiotics, (+)-catechin, (-)-epigallocatechin gallate, apoptosis, cancer, flow cytometry

Introduction

Plants produce secondary metabolites to defense against herbivores and pathogens. Once taken by living organisms, such natural substances become xenobiotics. Xenobiotics may exert numerous toxic effects in biological systems including human organism. However, several plant-derived natural substances have been proved to exert beneficial effects in mammals. Recently, a lot of effort has been made in order to understand the mechanisms of action of plant bioactive compounds in the human body. Growing evidence indicate that numerous phytochemicals may exert beneficial effect in humans and may potentially be used against particular pathologies and diseases. Various epidemiological studies report the positive correlation between consumption of several nutrients and lower incidence of specific tumors, such as liver and colorectal cancer, which suggests that plant xenobiotics may activate specific anticancer mechanisms [1]. Since cancer is a growing problem in western-lifestyle societies, the easy-accessible, low-toxic and low-cost

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Contribution was presented during ECOpole'18 Conference, Polanica-Zdroj, 10-13.10.2018

cancer-preventive strategies for general public are highly required. Chemoprevention using naturally occurred xenobiotics has been considered as appealing and easy to implement strategy. However, evidence reports on the use of polyphenols in diet to reduce tumor in humans are ambiguous [2]. Recently, it has been shown that combinatory treatment may be an effective approach in combating tumor in humans [3]. Growing evidence suggests that some xenobiotics derived from plants may be used in combination with anticancer drugs [4]. Such a combinatory approach comprises the enhancement of the existing anticancer treatment and the reduction of side effects caused by a standard drug. Therefore, the detailed study on molecular mechanisms of action of particular plant xenobiotics are necessary. Recent data indicate that catechins might be one of the potent group of naturally occurred compounds.

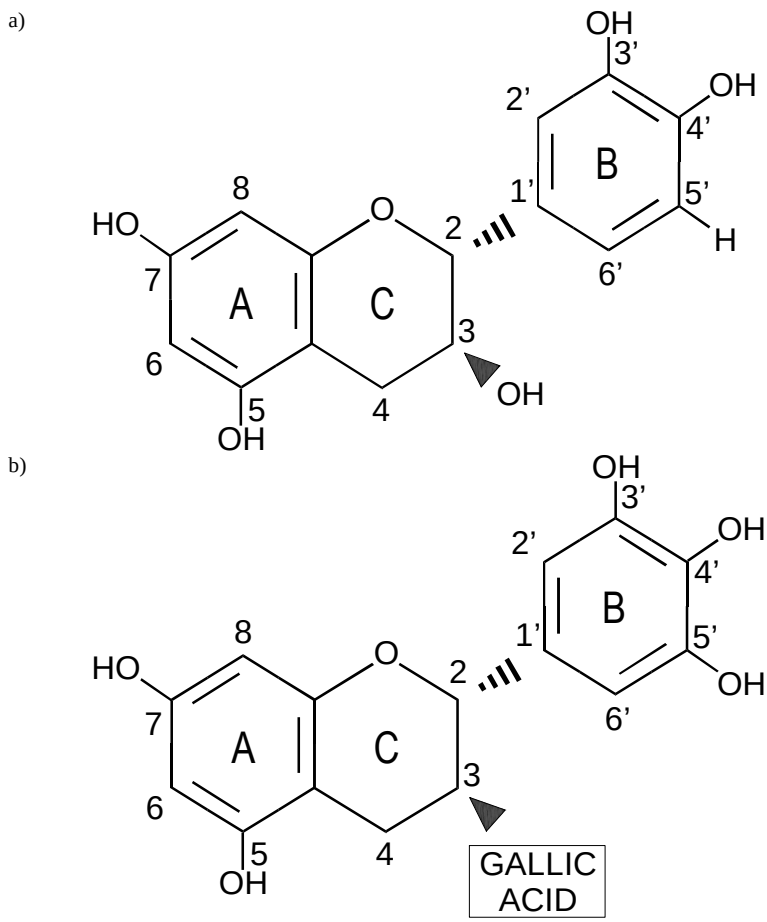

Fig 1. Chemical structures of: a) (+)-catechin and b) (-)-epigallocatechin gallate

Catechins are abundantly found in the plant *Camellia sinensis*. Especially beverages including black tea, oolong tea, green tea, and white tea are rich in catechins. These group of compounds are also found in cocoa, fruits, such as peaches, and in barley grains [5]. Catechins are a subclass of flavanols and are mainly represented by (+)-catechin ((2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol) and (-)-epigallocatechin gallate (EGCG; [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate), galloylated derivative of catechin. The chemical structures of compounds were presented in Figure 1. It was estimated that average daily infusion of green tea contains 1 g/dm^3 of compounds, however the fermentation may reduce the amount of catechins in particular beverage. Red wine and chocolate may considerably supply the daily intake of catechins [6]. Catechins have recently been studied due to their antioxidant activity. It was established that flavanols including catechins may pass through biological membranes. Although this group of phytochemicals is very abundant in plant foods, the bioavailability of the compounds is poor in humans [7]. However, some epidemiological studies have reported the beneficial influence of this compounds on human population. Especially, the leading role of catechins in the French paradox has been established [8]. Consistently, several pharmacological applications of catechins were proposed, especially in the prevention of metabolic disorders and as antiviral agents [9].

It was recognized that on molecular level, that (+)-catechin and (-)-epigallocatechin gallate exert their action by several mechanisms, especially by suppressing the enhanced inflammation process [10, 11]. The chronic inflammation has been linked to the increased risk of cancer, thus the attenuation of excessive synthesis of inflammatory cytokines is thought to be crucial in chemoprevention. What is more, the malfunction of cell death is one of the primal features of malignant cells and apoptosis is the most critical defense mechanism against cancer in organism. Several reports indicate that phytochemicals may induce apoptosis in tumor cells by influencing particular regulatory points in the apoptotic cascade as well as regulation of gene expression [12]. Many recent studies have suggested that catechins may efficiently modulate several signaling pathways within tumor cell thereby interrupting the process of carcinogenesis as well as tumor propagation [13]. Since the induction of programmed cell death is one of the most potent anticancer mechanisms, numerous drugs aim at apoptosis. Xenobiotics may cause damage to neoplastic cells *via* another mechanism called “necrosis”. However, within human body, necrosis may at the same time induce inflammation and such a way of tumor cells disposal may be less advantageous to organism than apoptosis [14].

The detection of biological activity of specific anticancer compounds can be carried out with a number of assays. In present experiments, flow cytometry technique was used, since it was of interest to distinguish between the number of apoptotic cells and necrotic cells in the population of tumor cells exposed to both xenobiotics. We tested whether (+)-catechin and/or (-)-epigallocatechin gallate may induce cancer cell death using *in vitro* tumor model of liver hepatoma cells.

Materials and methods

Chemicals

Catechin and (-)-epigallocatechin gallate were purchased from Sigma-Aldrich, Germany. Apoptosis/necrosis kit was purchased from Biotium, USA. Media and sera were from Lonza, Switzerland, antibiotics mixture and Trypsin - 0.05 % EDTA solution were from Gibco, USA. Sterile and non-toxic plates, flasks, tips, centrifuge tubes were from Sarstedt, Germany. Deionized water 18 Mohm.cm was obtained from Milli Ro & Q water purification system (Merck-Millipore, USA), ethanol was from Merck.

Cell culture

To examine the effect of (+)-catechin and (-)-epigallocatechin gallate Morris hepatoma 7777 cells from *Rattus norvegicus* (ATCC designation: McARH7777, CRL1601) were used. The cells were grown as monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Bovine Serum and with 1 % antibiotic solution (100 IU/cm^3 penicillin, 0.1 mg/cm^3 streptomycin). The cells were kept under standard cell culture conditions at 37 °C in a humidified atmosphere of 5 % of CO_2 in air. During the culture, the viability of cells was controlled with Trypan Blue Exclusion Test using automatic cell counter (Countess, Invitrogen). The morphology of cell culture was investigated by an inverted light microscope (Olympus IX-70 microscope, Olympus, Germany).

Cell apoptosis and necrosis assay

The analysis of apoptotic/necrotic/alive cells after 24 h incubation with catechins was performed with FACSCanto10C flow cytometer, using BD System Software (BD FACSDIVA V8.0.1, BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Fluorescent dyes Annexin-V (excitation/emission 490/515 nm) and Ethidium homodimer (EthD-III, excitation/emission 528/617 nm) were used for measurements. The cells were gated according to forward (FSC), side scatter (SSC), and appropriate fluorescence parameters. The living cells were defined as negative for Anexin-V and EthD-III, the apoptotic cells consisted of Anexin-V positive/EthD-III negative cells (early apoptosis) and Anexin-V/EthD-III positive cells (late apoptosis); the necrotic cells were Anexin-V negative and EthD-III positive. The results were given as the percentage of apoptotic or necrotic cells of the total counted cells.

Data analysis

The flow cytometry experimental data was shown as mean ± SD. Analysis was performed using one-way ANOVA followed by a Duncan post-hoc test. p values < 0.05 were considered statistically significant. Statistical calculations were carried out using the commercially available packages Statistica v10 (StatSoft, Inc., Tulsa, USA).

Results

The exposition of Morris hepatoma 7777 cells culture to (+)-catechin and (-)-epigallocatechin gallate, both at 0.1 mM/dm^3, slightly changed the morphology of cells, while each compound used at 0.5 mM/dm^3 visibly aggravated cell morphology, as observed under phase contrast microscope and shown in microphotographs (Fig. 2).

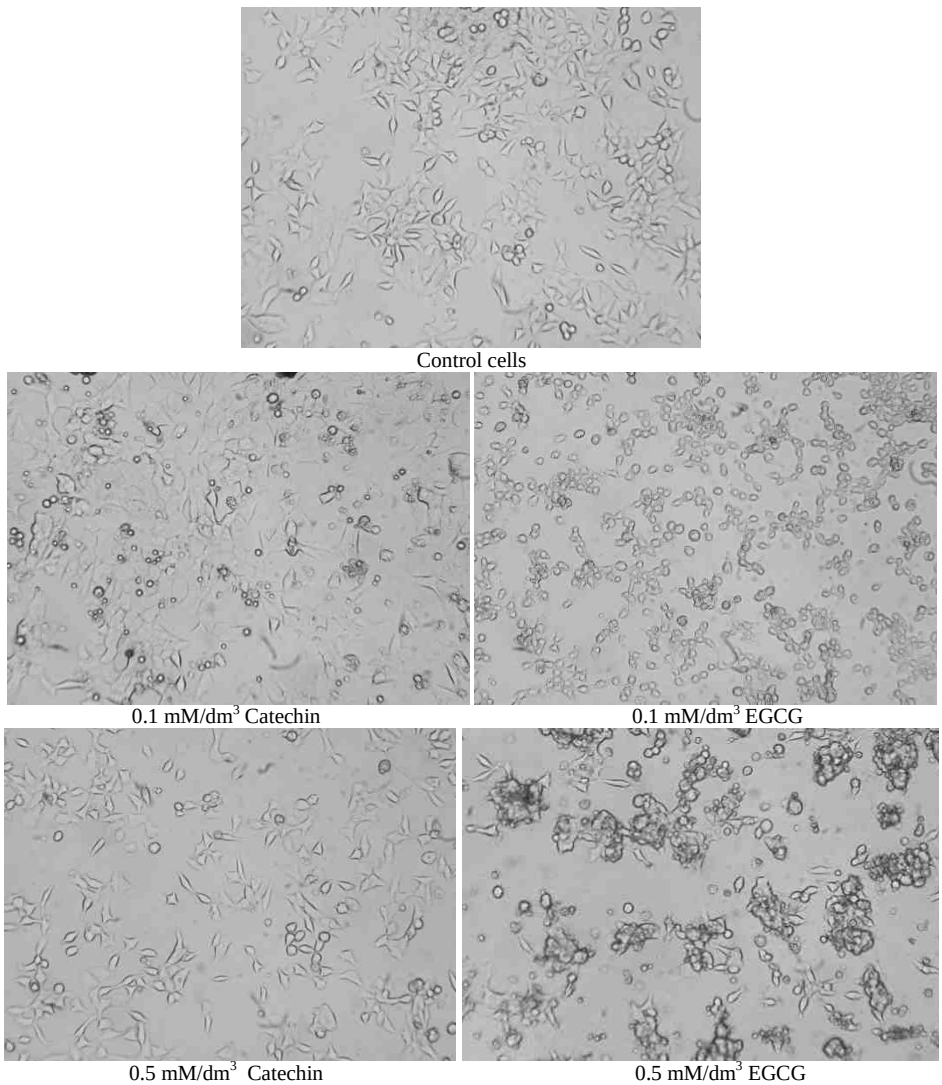

Fig. 2. The influence of (+)-catechin (A) and (-)-epigallocatechin gallate (EGCG) on morphology of Morris hepatoma 7777 cells. The cells were incubated for 24 h with selected doses of xenobiotics and the morphology of cell culture was investigated with an inverted light microscope

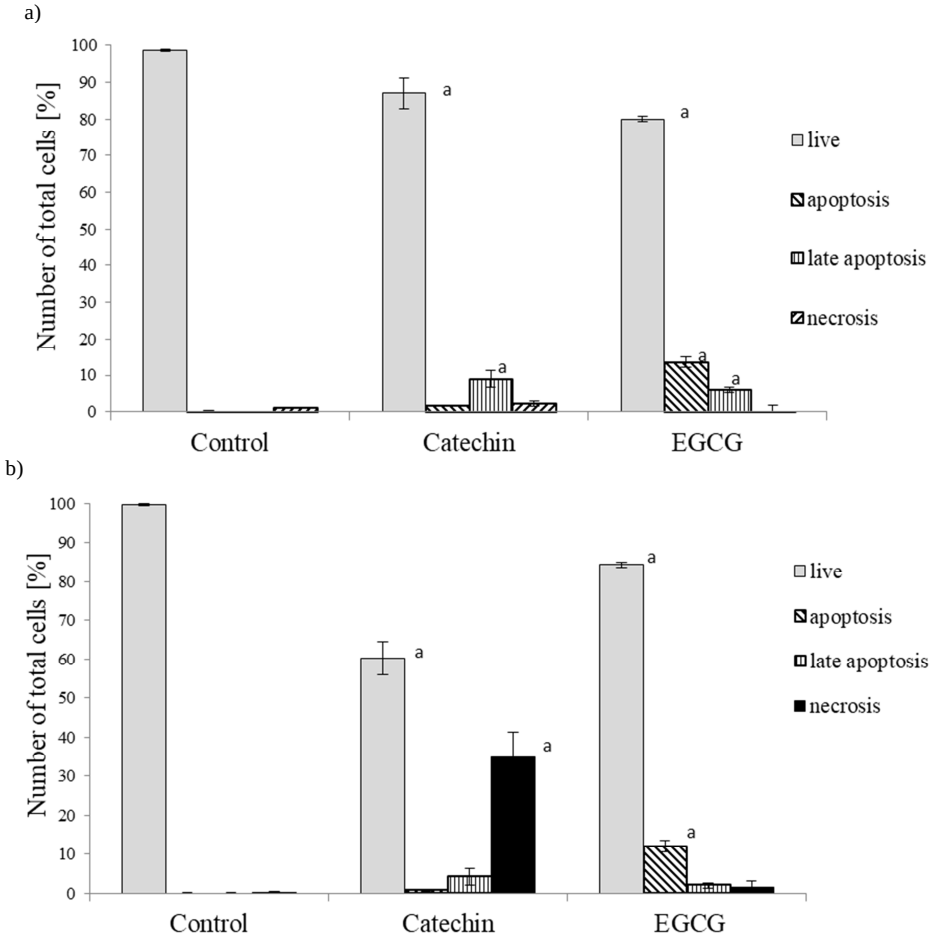

Fig. 3. (+)-catechin (A) and (-)-epigallocatechin gallate (EGCG) induce apoptosis/necrosis in culture of Morris hepatoma 7777 cells. The cells were incubated for 24 h with polyphenols at: a) 0.1 mM/dm^3 or b) 0.5 mM/dm^3 (^{a} $p < 0.05$ vs. control, $n = 3$)

These changes were followed by shifts in cell population towards apoptosis and necrosis. We found that treatment of Morris hepatoma 7777 cells with lower doses (0.1 mM/dm^3 of (+)-catechin and 0.1 mM/dm^3 of EGCG) caused decrease in the alive cells in both populations (Fig. 3a) ($p < 0.05$ vs. control). The presence of 0.1 mM/dm^3 EGCG in culture medium increased percentage of apoptotic cells (early and late apoptotic), while exposition of cells to (+)-catechin caused rise in late apoptotic cells. The incubation of Morris hepatoma 7777 cells with the presence of both tested catechins at higher doses (0.5 mM/dm^3 of (+)-catechin and EGCG at 0.5 mM/dm^3) caused the explicit induction of cell death ($p < 0.05$ vs. control). However, while (+)-catechin inhibited cancer cells mainly

via necrosis, EGCG significantly induced the number of apoptotic cells in population, as shown in Figure 3b ($p < 0.05$ vs. control).

Discussion

To date, cancer treatment protocols in humans have been based on the application of single cytostatic drugs such as cisplatin (cis-diamminedichloroplatinum(II)) and 5-fluorouracil. However, the conventional therapies are usually toxic to whole organism, not only tumors, and resistance to drugs often occurs. Recently, the use of combined treatment of cytostatic drugs with other agents has been recommended as the most effective strategy against cancer [15]. Especially, the potential use of plant secondary metabolites is of interest. The plant xenobiotics may exert their effects by increasing programmed cell death, thereby decreasing the number of cancer cells within the body.

Recent studies have shown that (+)-catechin and (-)-epigallocatechin gallate may inhibit malignant transformation and/or retard the development of cancer cells in mammals [16]. It was established using *in vitro* models that the process of the elimination of tumor cells may be significantly enhanced by the action of several phytochemicals [17]. In present experiments we report that catechins may efficiently suppress Morris hepatoma 7777 cells performance, as measured using flow cytometry and observed under phase-contrast microscope. The incubation of tumor cells with (+)-catechin caused greater elimination of malignant cells from population than (-)-epigallocatechin gallate. However, it should be emphasized that (-)-epigallocatechin gallate acted mainly via apoptosis, while (+)-catechin caused necrotic damage to tumor cells. We may speculate that in *in vivo* conditions the use of the (-)-epigallocatechin gallate may eliminate cancer cells without causing excessive inflammation and possible damage of tumor surroundings tissues.

Growing body of evidence indicate that catechins exert anti-cancerogenic action towards various cancer cell lines by triggering apoptosis [18]. Moreover, catechins may selectively evoke neoplastic cell death, therefore these compounds may selectively kill malignant cells while normal cells can survive the treatment without any damages. In present study we report that (+)-catechin and (-)-epigallocatechin gallate have elicited cell death in liver hepatoma cells and in future experiments we plan to assess whether (+)-catechin and (-)-epigallocatechin gallate may influence the survival of normal cells. As mentioned, catechins may be very effective in *in vitro* studies while their action within the body is less efficient. In terms of increasing bioavailability of polyphenolic compounds, several modifications of (+)-catechin chemical structure have been recently proposed in order to improve its selectivity and efficacy of anti-cancer action [19].

The assessment of novel mechanisms of plant-derived xenobiotics action in organism may help to support the existing treatment of cancer in humans. In current study we showed that catechins, especially and (-)-epigallocatechin gallate, may precisely act on programmed cell death. In view of recent findings catechins may be considered as potential agents for support therapeutic anticancer interventions.

Conclusions

Recently, the development of new therapies provides significant benefit to cancer patients. The application of plant secondary metabolites may counteract the adverse effects

of existing therapies and/or augment the action of standard drugs. In particular, the identification of new molecules that regulate signaling pathways and work against cancer development is of interest. In current study we present that (+)-catechin and (-)-epigallocatechin gallate, xenobiotics abundantly found in plants, may be potentially used for targeting apoptosis in cancer cells. However, a detailed knowledge of mechanisms that activate apoptotic pathways in tumor as well as normal cells is required. The present work demonstrated that both catechins may elucidate death of tumor cells and while (+)-catechin exerts its effect *via* necrosis, (-)-epigallocatechin gallate eliminates malignant cells mainly *via* apoptosis. We conclude that the use of catechins, especially (-)-epigallocatechin gallate, in combination with anticancer agents that trigger apoptosis in cancer cells may be a path forward for the development of more effective therapies for cancer patients.

Acknowledgments

This work was supported by K/ZDS/007965 project (Faculty of Pharmacy, Jagiellonian University Medical College, Krakow, Poland).

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Abstrakt: W środowisku występują substancje obce o charakterze ksenobiotyków, czyli związków chemicznych niesyntetyzowanych w sposób naturalny w ciele człowieka. Wiele takich aktywnych biologicznie związków może być produkowanych przez rośliny. Przeprowadzone dotychczas badania wskazują, że drugorzędowe metabolity roślinne mogą wywierać pozytywny wpływ na zdrowie człowieka. Na przykład wyniki ostatnich obserwacji potwierdziły chemoprewencyjne właściwości katechin. (+)-katechina (*ang. (2R,3S)-2-(3,4dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol*) oraz (-)-galusan epigallokatechiny (*ang. EGCG; [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate*) to związki o charakterze ksenobiotyków występujące w diecie człowieka. Ostatnie odkrycia zwracają uwagę w szczególności na proapoptotyczne właściwości związków z tej grupy. Obydwa wymienione związki chemiczne mogą modyfikować przebieg wewnątrzkomórkowych ścieżek sygnałowych w komórkach nowotworowych. Takie oddziaływanie może uruchamiać kaskadę aktywności prowadzącą do apoptozy, programowanej śmierci komórki neoplastycznej. Apoptoza jest jednym z mechanizmów, które wykorzystuje się w nowoczesnych terapiach przeciwnowotworowych. Apoptozę charakteryzuje szereg zmian w przebiegu procesów biochemicznych i morfologii komórki. Komórki nowotworowe mogą być eliminowane również na drodze nekrozy, jednakże ten typ śmierci komórkowej często prowadzi do lokalnego stanu zapalnego, co jest zjawiskiem niekorzystnym dla organizmu. Drugorzędowe metabolity roślinne, takie jak (+)-katechina i (-)-galusan epigallokatechiny, mogą wykazywać zdolność do wywoływania śmierci apoptotycznej komórek nowotworowych i potencjał ten może być wykorzystywany w nowoczesnych przeciwnowotworowych terapiach kombinowanych lub do wspierania istniejącego leczenia.

Słowa kluczowe: ksenobiotyki, (+)-katechina, (-)-galusan epigallokatechiny, apoptoza, nowotwory, cytometria przepływowa