Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2015; 7(2); 108-115

ISSN: 0975-5160

Research Article

Cytotoxic Action of Flavonoids in Human Burkitt`S Lymphoma Cell Lines and its Antiandrogenic Modulation

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Available Online: 15th March, 2015

ABSTRACT

In the past decades, studies of anticancer agents from natural sources have attracted a great attention whereas flavonoids are considered as interesting lead compounds. Differently from various leukemia cells, the knowledge about the cytotoxic action of these plant secondary metabolites in lymphoma cells is much scarcer. Therefore, the antiproliferative activity of structurally different flavonoids (baicalein, luteolin, chrysin, quercetin, fisetin, hesperetin) was described in two human Burkitt`s lymphoma lines, Daudi and Namalwa, showing baicalein as the most potent agent among the tested compounds (IC_{50} values of 6.97 μM and 23.71 μM, respectively). Other flavonoids revealed cytotoxic effects in higher concentration range whereas hesperetin displayed no activity even at 100 μM. In Daudi, but not in Namalwa cells, the antiproliferative action of baicalein and luteolin was partially suppressed by coincubation with 1 μM antiandrogen hydroxyflutamide showing that androgen receptor-related signaling pathways might be involved in the cytotoxicity of these flavones. Androgen receptors are indeed expressed in Daudi, but not in Namalwa cells. However, at higher micromolar doses, hydroxyflutamide itself induced the growth inhibitory effects in both cell lines. Therefore, as flavonoids offer novel potential leads for antilymphoma therapy, the possible involvement of gender-specific mechanisms in their cytotoxic action certainly needs further investigation.

Keywords: Burkitt`s lymphoma; Flavonoids; Cytotoxicity; Androgen receptor; Hydroxyflutamide

INTRODUCTION

Hematologic malignancies, including leukemia, lymphoma and myeloma, constitute about 9% of all new cancer cases being the forth most frequently diagnosed neoplasms in economically developed countries[1]. Lymphoma stems from the lymphatic tissue and comprises two major forms, Hodgkin lymphoma and non-Hodgkin lymphoma[2]. Burkitt`s lymphoma (or small non-cleaved cell lymphoma) is a type of non-Hodgkin lymphoma that affects the B-cells[3,4]. It is a highly aggressive malignancy being considered as the fastest growing human cancer[3,5-7]. This disease is characterized by the diffuse infiltration of intermediately sized B-cells into nodal and extranodal tissues and the tumor is associated with Epstein-Barr virus[5].

In the last three decades, the global incidence of lymphoma has steadily raised[8]. Burkitt`s lymphoma affects both adults and children manifesting more commonly in men than in women. Although this disease is sensitive to chemotherapy, immunotherapy and/or molecularly targeted strategies, the different treatment schemes reveal varied success rates and the outcome is generally dependent both on the stage of malignancy as well as the group of patients being worse in children and patients with coexistent AIDS[6-8]. This means that the antilymphoma therapy presents still a major challenge and novel effective agents with fewer adverse effects are highly desirable. Therefore, further studies to develop novel therapeutic strategies for improvement of palliative care and prolonging life expectancy of lymphoma patients are urgently needed[2,7,8].

Numerous reports have shown that an increased and regular consumption of plant-based diet may reduce the risk of cancer as different phytochemicals can prevent the initiation and promotion events related to carcinogenesis[9,10]. Therefore, many studies are currently focused on the finding of novel bioactive compounds from natural sources and a large number of natural products is already evaluated according to their potential chemopreventive and chemotherapeutic properties[4,10,11]. Indeed, several compounds exerting anticancer activity *in vitro* and *in vivo* models are isolated from plants[4], whereas flavonoids represent one of the most interesting and widely distributed groups of such agents. These plant secondary metabolites are polyphenolic compounds displaying a broad spectrum of biological and pharmacological activities, including intervention with processes associated with carcinogenesis, such as antioxidative and free radical scavenging effects, antiproliferative action, antiangiogenic activity, induction of cell cycle arrest and apoptosis, and alteration of gene expression patterns[12-15]. Compared to various cell lines derived from different types of leukemia, the knowledge about the action of flavonoids in lymphoma cells is still much more scarce[16]. In this way, it has been reported that the plant-isolated rotenoid deguelin decreased the

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proliferation, induced apoptosis and arrested the cells in G0/G1 phase in human Burkitt`s lymphoma Daudi cells[4]; a prenylated flavonoid icaritin revealed cytotoxicity in Burkitt`s lymphoma Raji and P3HR-1 cells causing S phase arrest and inducing apoptotic death[7]; flavone wogonin inhibited the growth and increased apoptotic rates in Raji cells[2]; and flavone glycosides baicalin and scutellarin decreased the cell proliferation and elevated apoptosis in human Burkitt`s lymphoma CA46 and Namalwa cells, respectively[6,8]. Although sparse, these data indicate that polyphenolic flavonoids can reveal antilymphoma effects and it is clear that this issue certainly needs further and more systematic investigation.

In this work, we have tested the action of six structurally different flavonoids (three flavones, two flavonols and one flavanone) in two human Burkitt`s lymphoma cell lines, Daudi and Namalwa. The first of these B lymphoblastic cell lines is derived from the peripheral blood of 16-years-old Black male patient; the second line is isolated from the blood cells of 3-years-old female patient.

MATERIALS AND METHODS

Reagents

Cell culture medium RPMI 1640, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). Phenol red-free RPMI 1640 and dimethyl sulfoxide (DMSO) were obtained from Corning Life Sciences (Tewksbury, MA, USA). All flavonoids tested in this study (baicalein, luteolin, chrysin, quercetin, fisetin, and hesperetin) were from Santa Cruz Biotechnology (Dallas, Texas, USA). Estrogen receptor antagonist ICI 182780, androgen receptor antagonist hydroxyflutamide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibiotics ampicillin and kanamycin were from Applichem (St Louis, MO, USA). Reagents of the PCR reaction (Pfx buffer, Enhancer, 50 mM $MgSO_4$, 25 mM dNTP, and Pfx polymerase) as well as SYBR Green Dye were obtained from Invitrogen (Carlsbad, CA, USA). DNA ladder and DNA loading Dye were from Fermentas (Vilnius, Lithuania). Agarose was purchased from Naxo (Tartu, Estonia). PCR primers were synthesized in Metabion (Planegg/Steinkirchen, Germany).

Cell culture

The human Burkitt`s lymphoma cells Daudi and Namalwa were cultivated in suspension in a humidified atmosphere containing 5% CO_2 in air at 37^0C, in RPMI 1640 medium supplemented with 10% FBS, 0.1 mg/ml ampicillin and 0.05 mg/ml kanamycin. The cells were passaged twice per week.

Cytotoxic assay

The antiproliferative effects of flavonoids against Daudi and Namalwa cells were detected by the MTT colorimetric assay first described by Mosmann[17]. In detail, the cells were plated on to 96-well culture plates at concentration of $5x10^5$ cells/ml of medium, putting 100 µl of suspension to each well. As phenol red can interfere with the reading of absorbance[18], the cells were seeded in the phenol red-free RPMI 1640 medium. After overnight growth, cells were treated with varying doses of flavonoids (10 nM-100 µM) for additional 48 h. At the end of the treatment, 50 µl of MTT solution in PBS (5 mg/ml) was added to the wells and the plates were further incubated for 4 h followed by centrifugation and removing of the supernatant. The purple formazan crystals were dissolved with 150 µl DMSO and the absorbance at 540 nm was measured using a spectrophotometric plate reader. To calculate the proportion of surviving cells, the following formula was used: (OD of drug-treated sample – OD of blank)/(OD of control – OD of blank) x 100%, where OD of blank represents the absorbance reading of wells containing the buffer only (without cells) and OD of control represents the reading value of wells without any added test compounds. Dose-response curves were constructed to determine the half-maximal inhibitory concentrations (IC_{50} values). All separate experiments were performed in triplicates.

DNA isolation and polymerase chain reaction (PCR)

Genomic DNA was isolated using the AllPrep DNA/RNA Mini Kit of Qiagen (Chatsworth, CA, USA) according to the protocol of manufacturer and was stored at -20 ^{0}C. The PCR reaction was carried out in the volume of 25 µl, containing 1 µl of DNA, 2.5 µl of 10 x Pfx buffer, 2.5 µl of 10 x Enhancer, 0.5 µl of 50 mM $MgSO_4$, 0.3 µl of 25 mM dNTP, 1 µl of 100 µM primer solutions, and 0.2 µl of Pfx polymerase. The sequences of PCR primers were derived from the work of Walton et al. (2009) and were the following: androgen receptor (AR) upstream primer 5`-TTG GAT GGC TCC AAA TCA C-3´ and downstream primer 5`-GCA ATG ATA CGA TCG AGT TCC-3`, 148-bp product; estrogen receptor-α (ERα) upstream primer 5`-TGG GCT TAC TGA CCA ACC TG-3` and downstream primer 5`-CCT GAT CAT GGA GGG TCA AA-3`, 108-bp product; estrogen receptor-β (ERβ) upstream primer 5`-AGA GTC CCT GGT GTG AAG CAA-3` and downstream primer 5`-GAC AGC GCA GAA GTG AGC ATC-3`, 143-bp product[19]. The PCR reaction was performed as follows: 1 cycle of 5 min predenaturation at 94 ^{0}C, then 45 cycles of 30 s at 95 ^{0}C, 30 s annealing at 60 ^{0}C, 30 s extension at 72 ^{0}C, and followed by 1 cycle of 5 min extension at 72 ^{0}C. PCR products were analyzed by agarose gel electrophoresis and visualized by SYBR Green staining.

Statistical analysis

All data were treated using the GraphPad Prism statistical software. $P<0.05$ was considered statistically significant.

RESULTS

Cytotoxicity of flavonoids in human Burkitt`s lymphoma cell lines Daudi and Namalwa

The effects of six flavonoids (baicalein, luteolin, chrysin, hesperetin, quercetin, and fisetin) on the growth of Daudi and Namalwa cells were tested by incubating the cells with compounds for 48 h. The stock solutions of flavonoids (30 mM) were prepared in DMSO; all further dilutions were made in the phenol red-free cell culture

Table 1. Half-maximal inhibitory concentrations (IC_{50} values) of flavonoids in human Burkitt`s lymphoma cell lines Daudi and Namalwa. IC_{50} values were calculated from three separate experiments performed in triplicates

Flavonoids		IC_{50}, μM	
		Daudi	Namalwa
Baicalein	*Flavones*	6.97	23.71
Luteolin		19.99	31.55
Chrysin		40.46	71.78
Hesperetin	*Flavanone*	NA up to 100 μM [a]	NA up to 100 μM [a]
Quercetin	*Flavonols*	14.03	58.08
Fisetin		30.76	51.17

[a] NA, not active

Table 2. Effects of 1 μM antiestrogen ICI 182780 and 1 μM antiandrogen hydroxyflutamide on cytotoxic activity of baicalein and luteolin in human Burkitt`s lymphoma cell lines Daudi and Namalwa

Flavonoids		IC_{50}, μM	
		Daudi	Namalwa
Baicalein		6.97	23.71
	+1 μM ICI 182780	6.84	24.60
	+ 1 μM hydroxyflutamide	10.28	23.82
Luteolin		19.99	31.55
	+1 μM ICI 182780	18.92	29.99
	+ 1 μM hydroxyflutamide	28.05	31.41

medium. The highest dose of DMSO in the cell suspension was 0.1%, exerting itself no effect on the cellular viability of both Daudi and Namalwa cells as tested in the control experiments. However, all three flavones and two flavonols inhibited the cell growth with baicalein being the most active flavonoid (IC_{50} values of 6.97 μM and 23.71 μM in Daudi and Namalwa cells, respectively). The activity profile of tested flavonoids was baicalein>quercetin>luteolin>fisetin>chrysin in Daudi cells and baicalein>luteolin>fisetin>quercetin>chrysin in Namalwa cells. The respective dose-response curves are presented in Fig. 1 and the half-maximal inhibitory concentrations are listed in Table 1. The only flavanone tested, hesperetin, revealed no cytotoxic activity even at the highest tested concentration (100 μM) in both cell lines (Fig. 1).

As can be seen in Table 1, all cytotoxically active polyphenols were more potent towards Daudi cells compared to Namalwa cells. The respective sensitivity coefficients (i.e., the ratios of IC_{50} values in Namalwa cells to IC_{50} values in Daudi cells) remained in the range of 1.6-4.1. It means that despite being isolated from the patients with the same diagnosis (Burkitt`s lymphoma), Daudi cells are significantly more sensitive to flavonoids than Namalwa cells.

Expression of estrogen and androgen receptors in Daudi and Namalwa cells

One important difference between two cell lines used in this study is the fact that Daudi cells are derived from the peripheral blood of a male patient whereas the origin of Namalwa cells is the blood sample of a female lymphoma patient. Based on this knowledge it was exciting to examine the possible differences in the expression of gender-related hormone receptors in these blood cells. For this purpose, genomic DNA from both cell lines was isolated and analyzed in respect to the possible expression of estrogen receptors (ERα and ERβ) and androgen receptor (AR). The results of these PCR assay clearly indicated a substantial difference in the presence of gender-specific steroid receptors in these two cell lines. As depicted in Fig. 2, both ERα and AR, but not ERβ, were detected in Daudi cells, whereas no products of any of these receptors were amplified in Namalwa cells. These results together with the data of cytotoxic experiments indicate that gender specific hormonal receptors could be potentially involved in the antiproliferative action of flavonoids and warrant additional studies to further test this hypothesis.

Effect of antiestrogen ICI 182780 and antiandrogen hydroxyflutamide on cytotoxicity of flavonoids

To clarify the potential role of ERα and AR in the higher sensitivity of Daudi cells towards flavonoids and to study the possible involvement of these receptors in the signal transduction pathways leading to cytotoxic responses, the effects of inhibitors of estrogen receptors (ICI 182780) and androgen receptor (hydroxyflutamide) were tested. For this aim, the dose response curves of two most active flavonoids (baicalein and luteolin) were measured by coincubating the cells with 1 μM antiestrogen or 1 μM antiandrogen and the respective IC_{50} values were calculated. The doses of inhibitors (1 μM) were chosen high enough to assure the full suppression of the activity of respective receptors[20,21].

As supposed, neither of the antiendocrines influenced the dose response curves of baicalein and luteolin in Namalwa cells where no respective receptors were detected (see Table 2). Also, the addition of 1 μM ICI 182780 had no effect on the potency of these flavones in Daudi cells, regardless of the presence of ERα in these cells (Fig. 2). These data indicate that the growth inhibitory action of baicalein and luteolin did not involve the components of estrogen receptor-related signaling pathways (Table 2). However, the presence of 1 μM hydroxyflutamide induced the small but statistically significant shift of the concentration-activity curves of baicalein and luteolin towards the higher doses, increasing also the respective IC_{50} values (Table 2). These results demonstrate that the antiproliferative activity of tested flavones is partially mediated by the cellular signal transduction pathways mediated by androgen receptor and therefore, the intervention with these events can also affect the cytotoxic action of flavonoids.

Androgen receptor non-mediated antiproliferative activity of hydroxyflutamide in Burkitt`s lymphoma cells

To ascertain that the modulating activity of hydroxyflutamide on the cytotoxicity of baicalein and luteolin in Daudi cells could not be caused by the growth inhibitory effect of antiandrogen itself, the action of 1 μM hydroxyflutamide was tested alone. As seen in the MTT

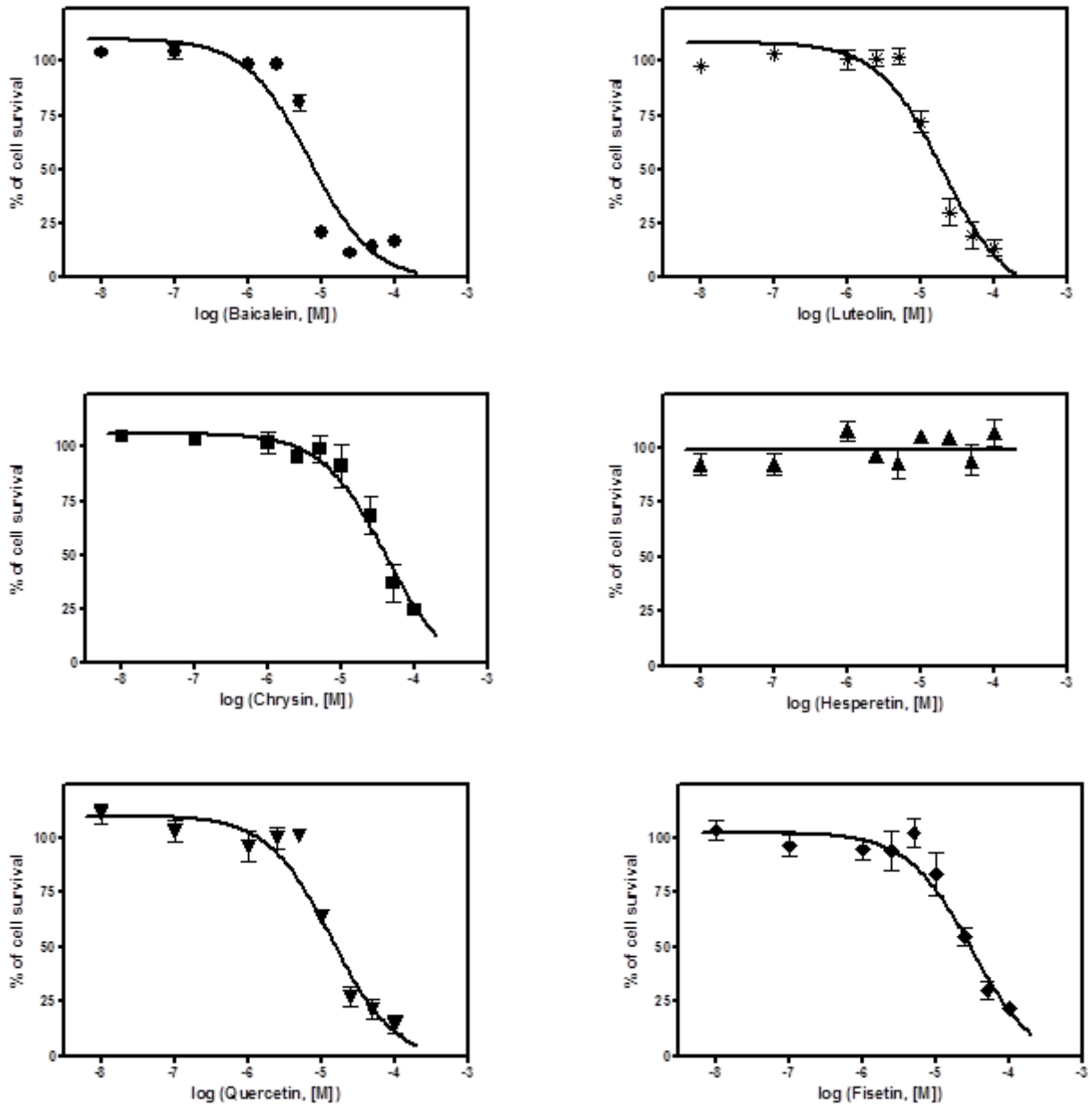

Fig. 1A Cytotoxic effects of baicalein (●), luteolin (*), chrysin (■), hesperetin (▲), quercetin (▼), and fisetin (♦) in human Burkitt`s lymphoma cell lines Daudi (a) and Namalwa (b). Cytotoxic effects were measured using the MTT assay by incubating the cells with flavonoids for 48 h. Dose-response curves were constructed from the data obtained from three separate experiments all performed in triplicates

reduction assay, hydroxyflutamide was completely inactive at this concentration (Fig. 3) showing that the modulating effect measured in Daudi cells was not caused by the direct decrease in cellular viability.

However, in the higher concentration range, i.e., at the doses significantly more than those acting on the androgen receptor, hydroxyflutamide itself exerted dose dependent cytotoxic activity in both human Burkitt`s lymphoma cell lines, Daudi and Namalwa, with the half-maximal inhibitory doses of 22.91 μM and 45.81 μM, respectively (see Fig. 3). By virtue of this higher concentration range, but also of the fact that there is no androgen receptor in Namalwa cells based on our PCR assay, these antiproliferative effects can not be mediated by androgen receptors. Thus, antiandrogen hydroxyflutamide displays cytotoxic activity in lymphoma cells by decreasing the cellular viability, and the respective molecular mechanisms as well as possible physiological meaning certainly need further investigation.

There was no effect of antiestrogen ICI 182780 on the growth of Daudi or Namalwa cells in the concentration range of 10 nM to 100 μM (data not shown).

DISCUSSION

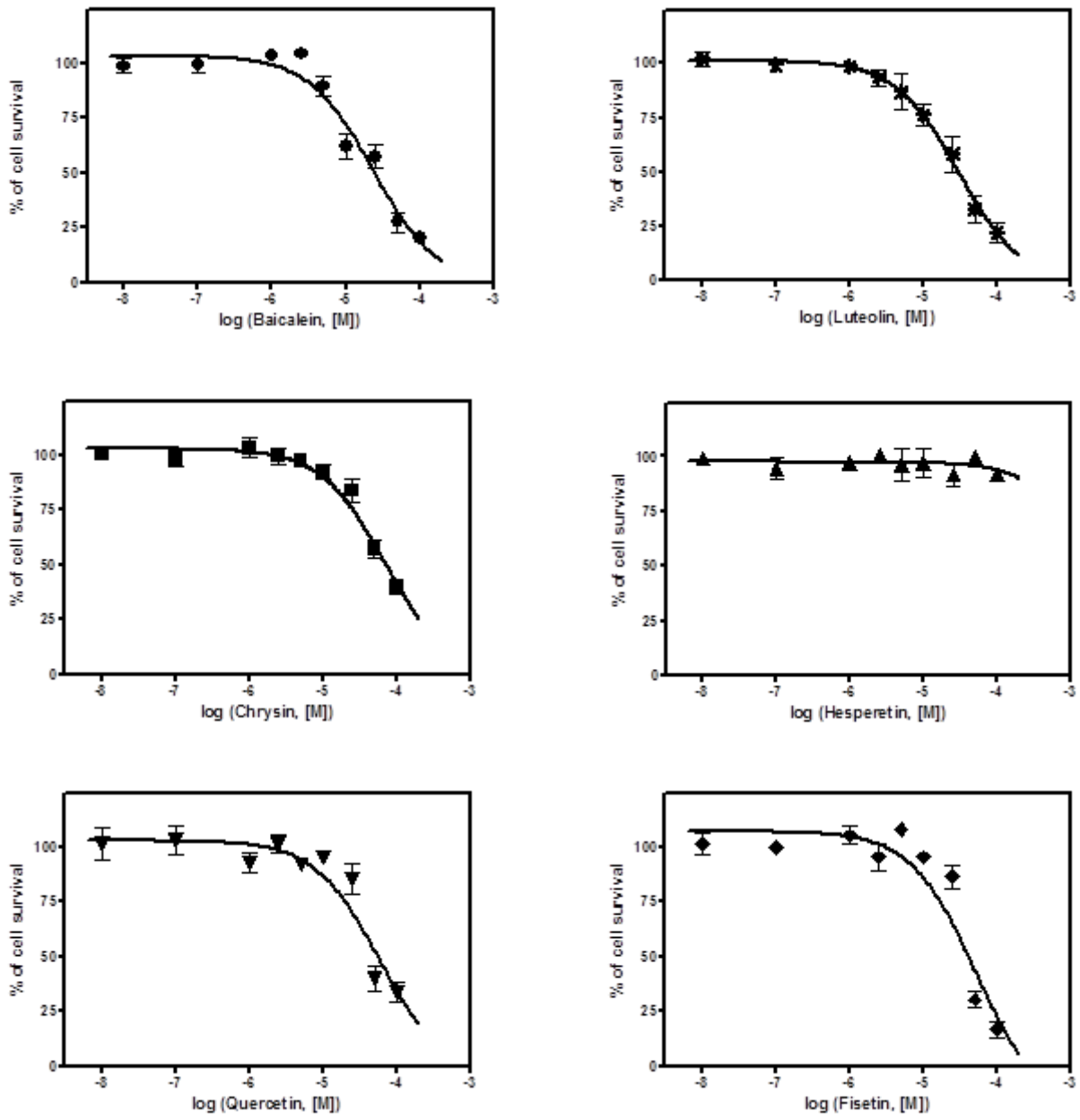

Fig. 1B Cytotoxic effects of baicalein (●), luteolin (*), chrysin (■), hesperetin (▲), quercetin (▼), and fisetin (♦) in human Burkitt`s lymphoma cell lines Daudi (a) and Namalwa (b). Cytotoxic effects were measured using the MTT assay by incubating the cells with flavonoids for 48 h. Dose-response curves were constructed from the data obtained from three separate experiments all performed in triplicates

Flavonoids represent a large class of dietary non-nutritive polyphenols widely distributed in fruits, vegetables, herbs, seeds, nuts, and some beverages like tea, and are categorized into different subgroups, including flavanols or catechins, flavonols, flavones, flavanones, isoflavones, and anthocyanidins[16,22]. These plant secondary metabolites display various biological properties which may contribute to the chemopreventive and chemotherapeutic effects, such as antiproliferative action, mitigation of oxidative damage, induction of cellular differentiation, promotion of apoptotic cell death, and modulation of cell cycle[12,23]. Considering the natural origination, anticancer activities and low toxicity of these compounds to normal cells, flavonoids have recently attracted a great attention as potential candidates of novel chemotherapeutic drugs[24].

In this work, the cytotoxic effects of three flavones (baicalein, luteolin and chrysin), two flavonols (quercetin and fisetin) and one flavanone (hesperetin) were studied in two human Burkitt`s lymphoma cell lines, Daudi and Namalwa. Baicalein is an active flavone naturally extracted from the root of Chinese herb *Scutellaria baicalensis* Georgi; luteolin can be abundantly found in celery, parsley, onion leaves, and peppers; chrysin is a component of honey and propolis; the rich products regarding quercetin include apples, citrus, red grapes,

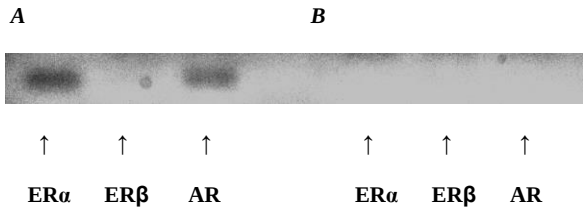

Fig. 2 PCR analysis of expression of estrogen receptors (ERα and ERβ) and androgen receptor (AR) in the human Burkitt`s lymphoma cell lines Daudi (a) and Namalwa (b)

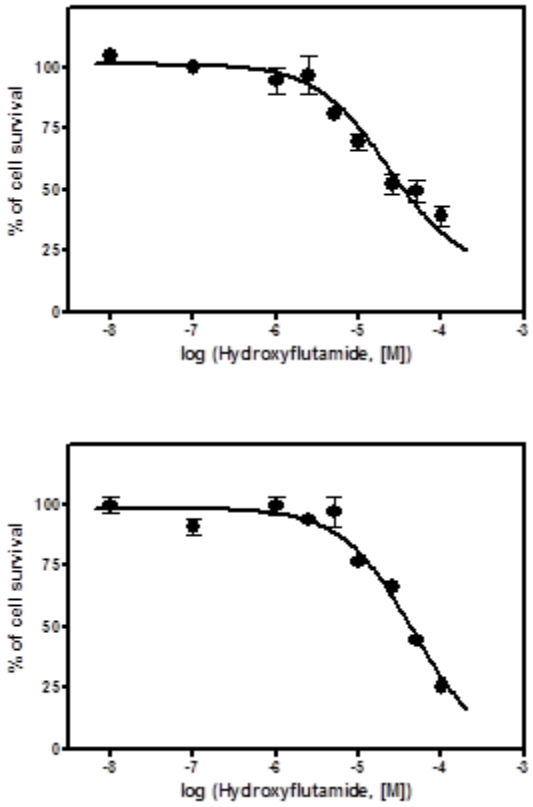

Fig. 3 Effect of antiandrogen hydroxyflutamide on the viability of human Burkitt`s lymphoma cell lines Daudi (a) and Namalwa (b). Cytotoxic activities were measured using the MTT assay by incubating the cells with hydroxyflutamide for 48 h. Each drug concentration was tested in triplicates. The calculated IC_{50} values were 22.91 μM and 45.81 μM for Daudi and Namalwa cells, respectively

cabbage, onion, and tomato; fisetin is mainly found in cucumber, onion, strawberry, apple, and kiwi; and hesperetin is a citrus flavonoid occurring in grapefruit and orange fruits[16,25].

All the tested flavones and flavonols induced cytotoxic effects in Daudi and Namalwa cells, whereas baicalein behaved as the most potent agent with the IC_{50} values of 6.97 μM and 23.71 μM, respectively. According to the National Cancer Institute, USA, a significantly cytotoxic phytochemical should have the IC_{50} value below 4 μg/ml or 10 μM[26], indicating that baicalein could be further considered as a potential antilymphoma agent. On the contrary, the only flavanone studied in this work, hesperetin, revealed no antiproliferative activity in either lymphoma cell lines even at the highest doses (i.e., up to 100 μM). Whether this feature is characteristic also to other members of flavanones subgroup certainly needs further analyses. Still, it is the first study where the cytotoxic profiles of a panel of flavonoids were tested and compared in different human lymphoma cell lines.

The antiproliferative activities of flavonoids in Daudi and Namalwa cells clearly demonstrated the significantly higher sensitivity of these natural compounds towards Daudi cells. As the origin of these two human Burkitt`s lymphoma lines differs regarding to the gender of the patients from whom the cells were originally derived and on the other hand, as it is well known that some flavonoids can interact with gender-specific binding sites[27,28], it was exciting to examine the expression of gender-related steroid receptors in these cells. Somewhat surprisingly, ERα and AR, but not ERβ, were detected in Daudi cells, whereas none of these receptors were present in Namalwa cells. Moreover, inhibition of androgen receptors (but not estrogen receptors) in Daudi cells resulted in a certain decrease in the cytotoxic potency of flavones, demonstrating that baicalein and luteolin can partially exert their antiproliferative activities through the androgen receptor-mediated signaling pathway. The precise mechanism of this event definitely needs additional studies. Currently, we know that Burkitt`s lymphoma affects more commonly men than women, and in USA, non-Hodgkin lymphoma constitutes the eighth leading cause of cancer mortality in males and the sixth in females[2]. The potential role of dietary flavonoids in this discrepancy and possible differential action of flavonoids in lymphoma cells of male and female origin should be further examined in *ex vivo* studies by testing the effects of these agents in malignant cells derived from the peripheral blood of lymphoma patients. At present, there is no data available about the antiandrogenic modulation of the cytotoxic activity of flavonoids in cancer cells; however, it was only recently demonstrated that the cytotoxic activity of genistein in human adult T-cell leukemia cells was exerted via an estrogen receptor-related pathway as this activity was eliminated by coincubation with antiestrogen ICI 182780[29].

Besides causing a small modulation of the cytotoxic action of flavones in androgen receptor-mediated manner in Daudi cells, antiandrogen hydroxyflutamide was itself able to induce the antiproliferative activity in higher concentration range, both in the AR-positive Daudi cells as well as the AR-negative Namalwa cells. The respective IC_{50} values were 22.91 μM and 45.81 μM. Further analyses should determine whether this effect is selective to lymphoma cells by studying the activity of hydroxyflutamide in other (blood) cancer cells and also in peripheral blood mononuclear cells from healthy donors. Also, the molecular targets of this antiandrogen in triggering the cytotoxic activity need more investigation. However, it is possible that the membrane-associated

androgen binding sites can be involved in the inhibition of growth of lymphoma cells by hydroxyflutamide[30].

In conclusion, in this work we have described for the first time the cytotoxic action of different flavonoids in two human Burkitt`s lymphoma cell lines Daudi and Namalwa by analyzing their activity profiles. These results showed the predominantly higher sensitivity of flavonoids towards Daudi cells. The antiproliferative effects of baicalein and luteolin was partially reduced by the coincubation of AR-positive Daudi cells with the low micromolar doses of antiandrogen hydroxyflutamide. No such effect appeared in the AR-negative Namalwa cells showing that in Daudi cells the growth inhibitory activity of baicalein and luteolin can be in part mediated via androgen receptor-related pathway. The cytotoxic effects of hydroxyflutamide at higher doses in both AR-positive and AR-negative lymphoma cells indicate that various structural derivatives of flutamide could be further tested towards the possible antilymphoma activity, but point also to the necessity to consider this action in the clinical treatment schemes where hydroxyflutamide is applied. It is self-evident that the respective mechanisms as well as the potential physiological implication of these events definitely require further research.

ACKNOWLEDGEMENTS

This work was supported by the grants of Estonian Research Council, No ETF8671, and University of Tartu, SARHOARENG.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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