# Dietary flavones enhance the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on bladder cancer cells

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KEY WORDS

bladder cancer ◗ TRAIL ◗ dietary flavones ◗ chemoprevention

ABSTRACT

**Introduction.** Flavonoids, naturally occurring in fruits and vegetables, demonstrate cancer chemopreventive properties. Flavones, a subclass of flavonoids, promote growth inhibition and apoptosis in bladder cancer cells. TRAIL plays an important role in immune surveillance and the defense mechanism against tumor cells. However, not all tumor cells are sensitive to TRAIL. TRAIL-resistant cancer cells can be sensitized to TRAIL-induced apoptosis by anticancer agents. We investigated the effect of TRAIL in combination with dietary flavones on bladder cancer cells.

**Materials and methods.** Two human bladder transitional cancer cell (TCC) lines: SW780 and RT112 were incubated with TRAIL and/or flavones (flavone, chrysin, apigenin, luteolin). Cytotoxicity was determined by MTT and LDH assays.

**Results.** Our study confirmed that RT112 bladder cancer cells were resistant to TRAIL, whereas SW780 cells were sensitive to TRAIL. We therefore examined the cytotoxic effect of TRAIL in combination with flavones on bladder cancer cells. We showed for the first time that flavones markedly augmented TRAIL mediated cytotoxicity against TCC cells. The strongest cytotoxic effect in combination with TRAIL was exhibited by luteolin. Co-treatment of bladder cancer cells with TRAIL and dietary flavones, especially luteolin, significantly sensitized bladder cancer cells to TRAIL induced cytotoxicity.

**Conclusion.** The tested flavones enhance the cytotoxic effect of TRAIL on bladder cancer cells. The obtained results suggest that dietary flavones (in particular luteolin) supported TRAIL-induced cytotoxicity in TCC cells and thus they might be promising chemopreventive agents in bladder cancer.

## INTRODUCTION

Bladder cancer is the fourth most common malignancy in men and eighth most common malignancy in women. Transitional cell carcinoma (TCC) is observed in more than 90% of pathologic subtypes of bladder tumors. Approximately 75-80% of bladder tumors present as non-muscle invasive (superficial) disease. Although most cases of bladder cancer are superficial, up to 70% of patients will have recurrence and 30% will progress to higher grade or stage [1, 2]. Because of the high frequency of tumor recurrence and the potential for progression to muscle-invasive disease, patients with superficial bladder cancer are closely monitored after their initial transurethral resection. This management involves regular cystoscopic evaluation and urine cytology analysis [1].

Chemoprevention is a rapidly growing area of uro-oncology that focuses on prevention of malignant disease using naturally occurring or synthetic agents. The term "chemoprevention" was first introduced by Dr. Michael Sporn, when he referred to the prevention of cancer development by natural forms of vitamin A and by its synthetic analogs [3, 4]. Bladder cancer represents an ideal tumor model for chemoprevention strategies. The urinary bladder is easily accessible and can be monitored by noninvasive or minimal invasive surveillance techniques. Preclinical and limited clinical data suggest that bladder cancer is responsive to efforts to delay or prevent its development in risk groups and in reducing recurrence in patients with established diseases. These facts indicate a great role of dietary phytochemicals that are useful in the interruption of bladder carcinogenesis and prevention of cancer recurrence [5-7].

The epidemiological evidence demonstrated that consumption of fruits and vegetables rich in flavonoids and phenolic compounds has been associated with reduced risk of several types of tumors [8, 9]. The experimental *in vitro* and *in vivo* studies confirmed the anticancer and chemopreventive activity of dietary flavonoids. Flavones belong to the flavonoids group, are widely distributed in the plant kingdom, and found as integral components of the human diet. Flavone, apigenin, chrysin and luteolin are found in celery, parsley, onions, broccoli, apples, oranges, cabbages, carrots, pepper, garlic, guava, tea, wine, basil, oregano, and propolis [8-10].

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) induces cell death in a wide variety of tumor cell lines and xenografts without causing toxicity to normal cells [11]. Soluble or expressed on immune cells (lymphocytes T, NK cells, neutrophils, monocytes and macrophages), the molecule TRAIL plays an important role in immune surveillance and defense mechanisms against tumor cells [11-13]. The naturally occurring TRAIL mediates apoptosis following binding to the two death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), on the cancer cells' surface [11, 14]. However, some tumor cells are resistant to TRAIL-mediated cytotoxicity. The decreased expression of TRAIL death receptors and pro-apoptotic proteins or increased expression of anti-apoptotic proteins in cancer cells are involved in TRAIL-resistance [14, 15]. We and others have shown that TRAIL-resistant bladder cancer cells can be sensitized by chemotherapeutic agents, ionizing radiation, or dietary flavonoids [15-17].

In this study we investigated the cytotoxic effects of TRAIL in combination with four flavones: flavone, chrysin (5,7-dihydroksyflavone), apigenin (5,7,4'-trihydroksyflavone), and luteolin (5,7,3',4'-tetrahydroksyflavone) on SW780 and RT112 bladder cancer cell lines. Flavones are one of the subclasses of flavonoids. Figure 1 presents the structures of flavones used in this study.

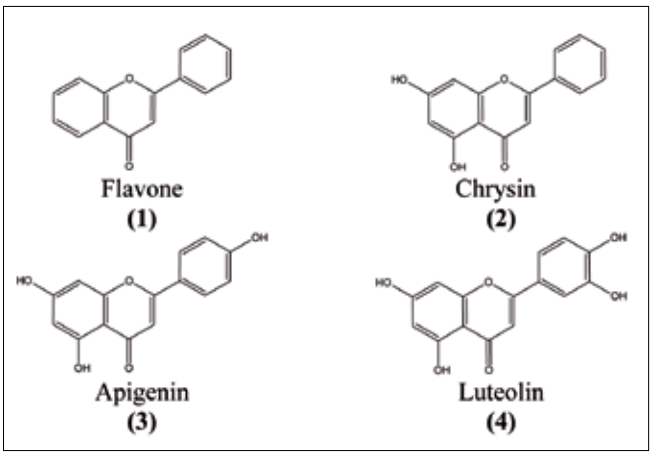


**Fig. 1.** Chemical structures of the studied flavones.

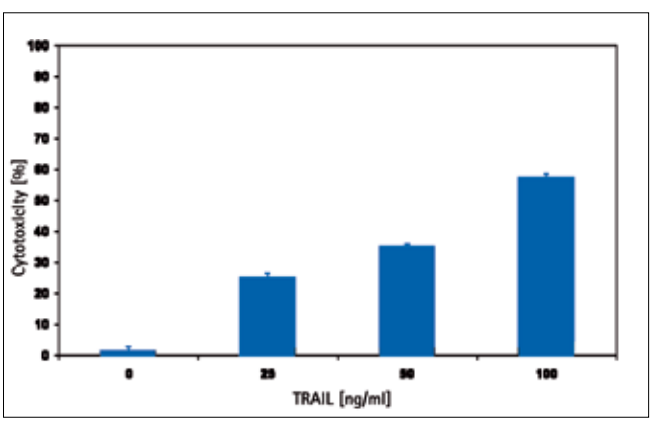


**Fig. 2.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml against SW780 bladder cancer cells.

## MATERIALS AND METHODS

### Bladder cancer cells

The tests were performed on two human bladder transitional cell cancer (TCC) lines derived from a bladder tumor obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH – the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and ATCC (American Type Culture Collection, Manassas, VA, USA):

SW780 cell line, cat. no. CRL-2169 – well differentiated transitional cells (G1),

RT112 cell line, cat. no. ACC-418 – moderately differentiated transitional cells (G2),

The bladder cancer cells were grown in monolayer cultures in plastic bottles of 70 ml and 500 ml (Nunc A/S Roskilde, Denmark): SW780 cells in Leibovitz's and RT112 cells in RPMI. All mediums were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C, SW780 cells in 100% air atmosphere, and RT112 cells in 95% air atmosphere and 5% $CO_2$. Reagents for a bladder cancer cell culture were purchased from PAA The Cell Culture Company (Pasching, Austria).

Cells adhering to the container bottom were trypsinized and suspensions were prepared for use during subsequent experiments. The number of bladder cancer cells tested in each experiment was $1x10^6$ per 1 ml of medium.

### TRAIL

Soluble, human recombinant TRAIL called "SuperKiller-TRAIL" [rhsTRAIL (CC-mutant)] and dilution buffer "Killer-TRAIL Storage and Dilution Buffer" were purchased from Alexis (San Diego, CA, USA).

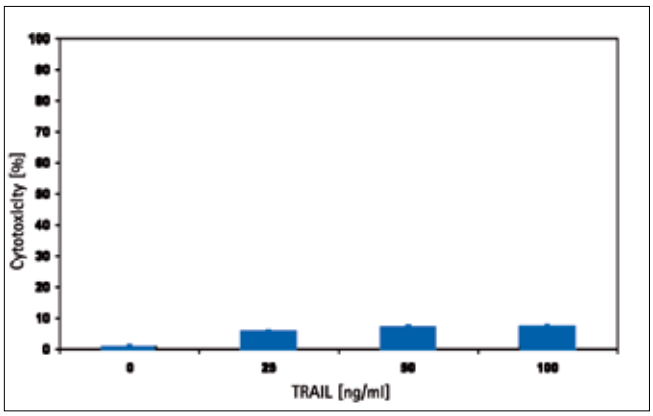


**Fig. 3.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml against RT112 bladder cancer cells.

### Flavones

The four flavones: flavone (1), chrysin (5,7-dihydroksyflavone) (2), apigenin (5,7,4'-trihydroksyflavone), (3) and luteolin (5,7,3',4'-tetrahydroksyflavone) (4) were purchased from Carl Roth GmbH (Karlsruhe, Germany) and Sigma Chemical Company (St. Louis, MO, USA).

### Cytotoxicity assays

### Mitochondrial dehydrogenase activity (MTT test)

The cytotoxic effects of tested agents on bladder cancer cells were assessed with a MTT test (bromo-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) involving the measurement of mitochondrial dehydrogenase activity [17-19]. The reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

### Lactate dehydrogenase activity (LDH test)

The cytotoxic effects of tested substances on bladder cancer cells were assessed by measuring lactate dehydrogenase (LDH) activity in the test by Roche Molecular Biochemicals (Mannheim, Germany) [20-22]. Lactate dehydrogenase is released from the cytoplasm into the culture medium as a result of cell membrane damage and cell lysis. The LDH activity increase in cell culture supernatants correlates with the rate of damaged cells (necrotic cells).

### Statistical analysis of results

The tests were performed in the same experimental conditions. The measurements were performed in four independent experiments. The significance level was $p \leq 0.05$.

The following standard tests were applied:

- the cytotoxicity assessment with regard to the different TRAIL levels – the Kruskal-Wallis one-factor analysis of variance,
- specific statistical analysis of the differences in cytotoxicity between two groups – the *U* Mann-Whitney test,
- analysis of simultaneous effects of two factors (cytotoxicity dependence on two factors: TRAIL and flavones) – a test based on a two-factor analysis of variance.

## RESULTS

### Cytotoxic effect of TRAIL on bladder cancer cells

The two bladder cancer cell lines were incubated with 25-100 ng/ml TRAIL for 24 hours. TRAIL induced cytotoxic effects on bladder cancer cells in a dose-dependent manner (Figs. 2 and 3). The cytotoxicity of TRAIL, at a concentration of 100 ng/ml, on SW780 cells

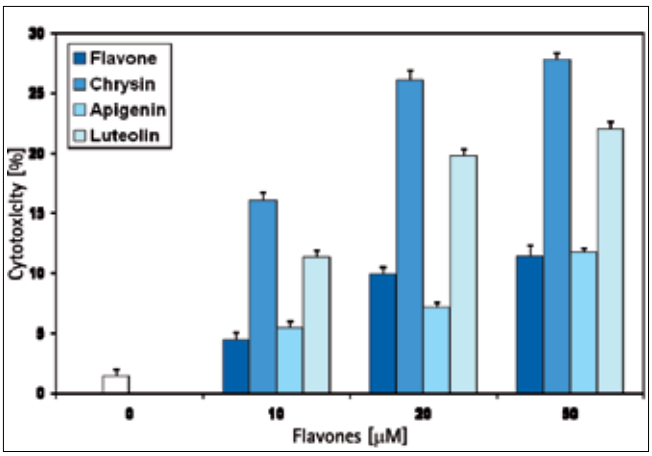


**Fig. 4.** Cytotoxic effect of flavones at concentrations of 10-50 μM against SW780 bladder cancer cells.

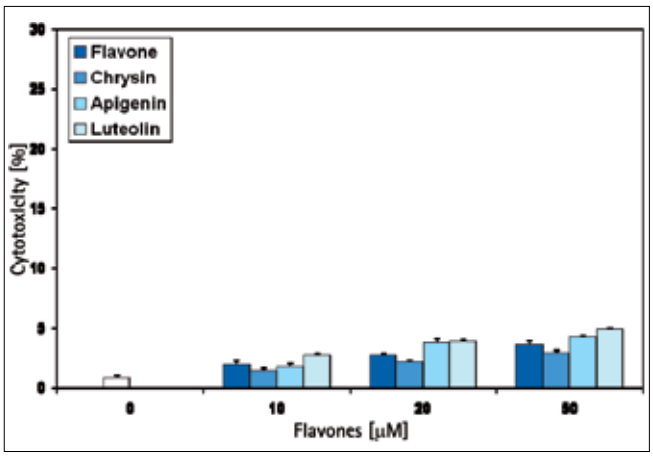


**Fig. 5.** Cytotoxic effect of flavones at concentrations of 10-50 μM against RT112 bladder cancer cells.

was 57.45 ±0.97%, and on RT112 cells it was 7.19 ±0.75. The obtained results indicated that SW780 cells were sensitive to TRAIL and the RT112 cells were resistant to TRAIL-mediated death. Ligand at the concentrations of 25-100 ng/ml did not induce lysis of bladder cancer cells, which was determined in the LDH test.

## Cytotoxic effect of flavones on bladder cancer cells

The bladder cancer cells were incubated with four flavones at the concentrations of 10-50 μM for 24 hours. The compounds induced cytotoxicity in bladder cancer cells in a dose-dependent manner (Figs. 4 and 5). Of the tested flavones the strongest cytotoxic activity exhibited chrysin and luteolin against SW780 cells (27.78 ±1.36% and 21.99 ±1.49% cell death respectively) and luteolin against RT112 cells (4.83 ±0.42). The SW780 bladder cancer cells in contrast to RT112 cells were also significantly susceptible to the cytotoxic effects of flavones.

## Cytotoxic effect of TRAIL in combination with flavones on bladder cancer cells

Next, we investigated cytotoxic effect of TRAIL in combination with flavones on bladder cancer cells. The cancer cells were

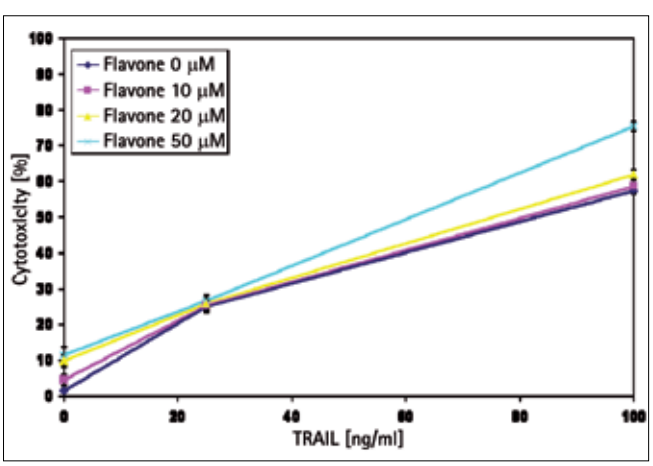


**Fig. 6.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with flavone at concentrations of 10-50 μM on SW780 bladder cancer cells.

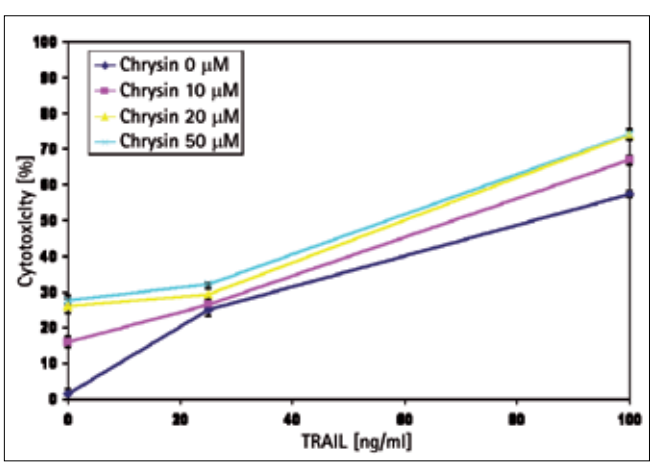


**Fig. 8.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with chrysin at concentrations of 10-50 μM on SW780 bladder cancer cells.

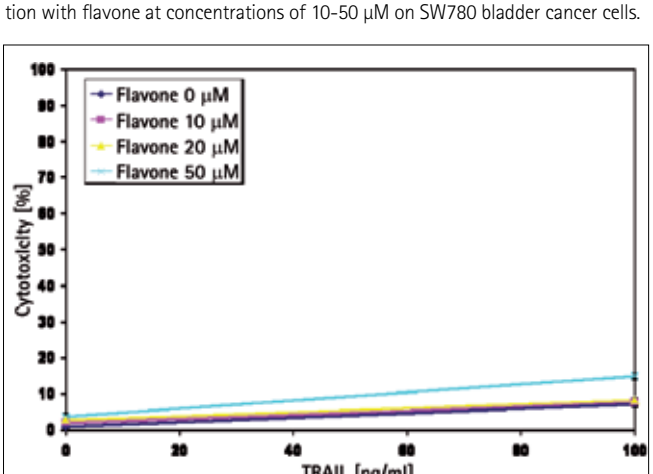


**Fig. 7.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with flavone at concentrations of 10-50 μM on RT112 bladder cancer cells.

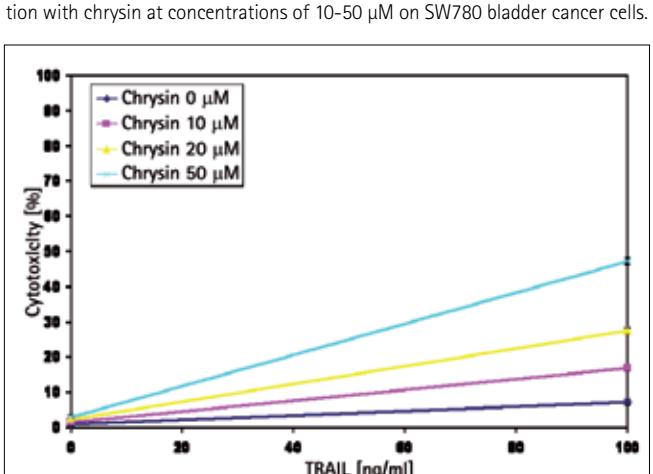


**Fig. 9.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with chrysin at concentrations of 10-50 μM on RT112 bladder cancer cells.

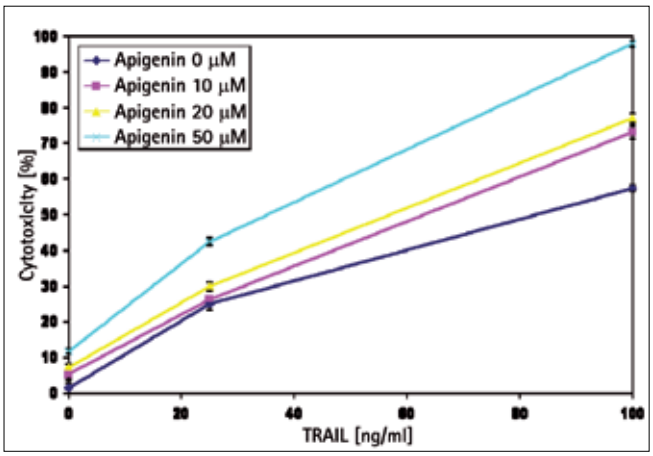


**Fig. 10.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with apigenin at concentrations of 10-50 μM on SW780 bladder cancer cells.

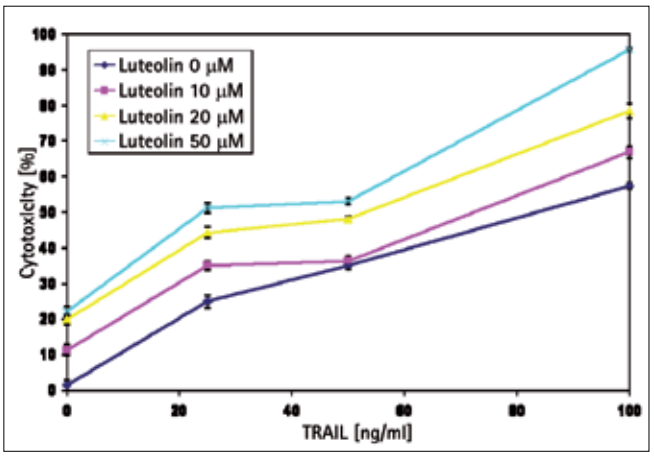


**Fig. 12.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with luteolin at concentrations of 10-50 μM on SW780 bladder cancer cells.

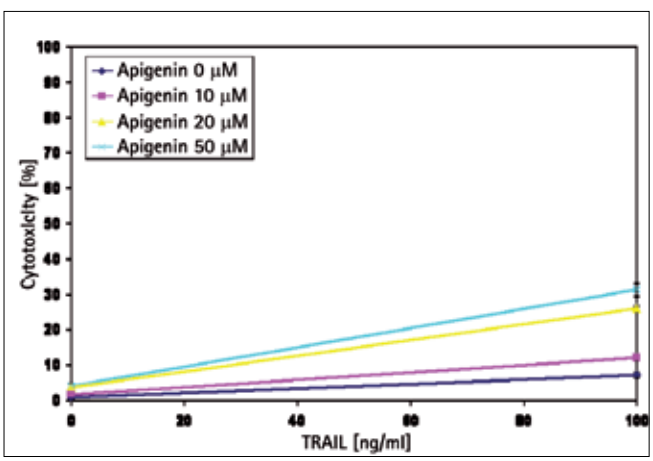


**Fig. 11.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with apigenin at concentrations of 10-50 μM on RT112 bladder cancer cells.

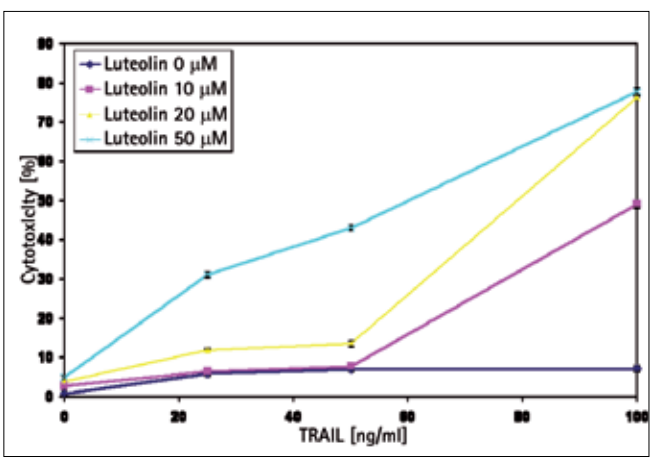


**Fig. 13.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with luteolin at concentrations of 10-50 μM on RT112 bladder cancer cells.

incubated with TRAIL at the concentrations of 25 ng/ml and/or 100 ng/ml and flavones at the concentrations of 10-50 μM for 24 hours. The co-treatment of SW780 cells with TRAIL and flavones increased the percentage of cell death to 75.49 ±1.37% for flavone, to 97.86 ±0.83% for apigenin, to 74.12 ±1.43% for chrysin, and to 95.71 ±0.38% for luteolin. The co-treatment of RT112 cells with TRAIL and flavones increased the percentage of cell death to 14.86 ±0.69% for flavone, to 31.40 ±1.87% for apigenin, to 47.23 ±0.97% for chrysin, and 77.72 ±0.88% for luteolin. All tested flavones augmented TRAIL-induced cytotoxicity in both bladder cancer cell lines (Figs. 6-13). The strongest cytotoxic activity in combination with TRAIL demonstrated luteolin and apigenin against SW780 cells and luteolin against RT112 cells. The flavones, especially luteolin, sensitized TRAIL-resistant RT112 cells to TRAIL-mediated cytotoxicity. The cytotoxic effects of TRAIL in combination with luteolin was demonstrated on both bladder cancer cell lines (Figs. 14 and 15).

## DISCUSSION

Many studies dealing with flavonoids have focused on their antioxidant and anti-inflammatory properties, but a number of reports in different cell lines, animal models and human epidemiological trials have pointed out an association between intake of dietary flavonoids and reduced risk of cancer, including bladder tumors [5, 6, 10].

The epidemiologic studies showed protective effects of diets rich in fruits and vegetables against cancer [4, 9]. The *in vitro* and *in vivo* experiments confirmed that flavonoids are the compounds responsible for these anticancer and chemopreventive effects [4, 8, 9]. Quercetin induced apoptosis, inhibited tumor growth and colony formation in EJ, J82, and T24 bladder cancer cell lines [23]. Epigallocatechin-3-gallate (EGCG) suppressed tumor growth and mediated programmed cell death in TCCSUP and T24 bladder cancer lines [24]. Chalcone arrested cell cycle progression and induced apoptosis in T24 and HT1376 bladder cancer cells and naringin in 5637 bladder cancer cells [25, 26]. Silibinin inhibited *in vivo* RT4 bladder cancer tumor xenograft growth [27].

Flavones exhibited cytotoxic activity against bladder cancer cells. Our study showed that flavones as the main subclass of the flavonoids induced cell death in bladder cancer. The cytotoxic effect of flavones was dependent on a tested compound and bladder cancer cell line. The strongest cytotoxicity against TCC cells exhibited chrysin and luteolin. The well differentiated transitional (G1) SW780 bladder cancer cells were more susceptible in contrast to moderately differentiated transitional (G2) RT112 cells. According to Ikemoto et al. three other flavones, baicalein, baicalin, and wogonin, possess antiproliferative activity against MBT2 and KU1 bladder cancer cells [28].

TRAIL plays an important role in the maintenance of immune homeostasis, host tumor surveillance, and defense against cancer cells [11, 13, 14]. However, some tumor cells are resistant to TRAIL-mediated cytotoxicity. We investigated the cytotoxic effect of TRAIL in combination with flavones in bladder cancer cells. Recombinant human TRAIL used in this study is a soluble protein based on

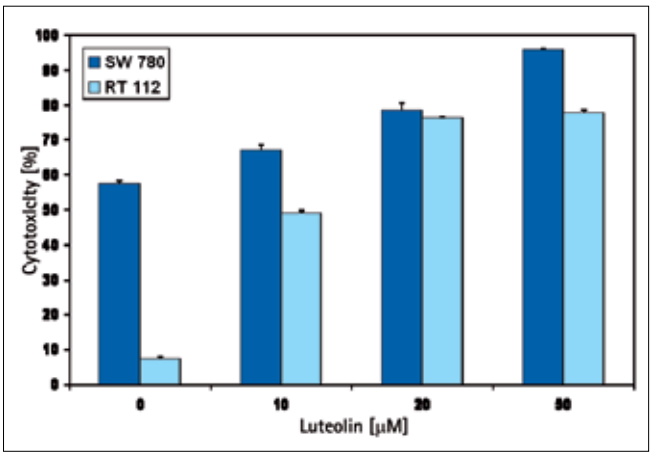


**Fig. 14.** Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with luteolin at concentrations of 10-50 μM on bladder cancer cells.

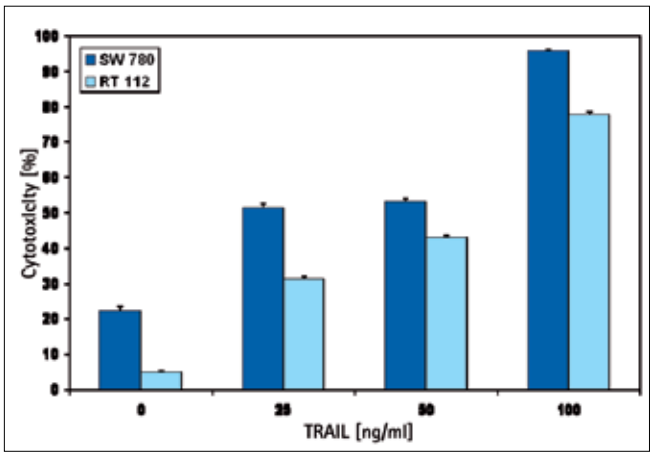


**Fig. 15.** Cytotoxic effect of TRAIL at concentrations of 25-100 ng/ml in combination with luteolin at the concentration of 50 μM on bladder cancer cells.

a natural ligand [12]. For the first time our results demonstrated that flavone, apigenin, chrysin, and luteolin augmented TRAIL-induced cytotoxicity in both bladder cancer cell lines. The flavones, especially luteolin, significantly overcame TRAIL-resistance in RT112 cells. The obtained data suggest the potentiating role of dietary flavones in anticancer immune effectors mechanisms with TRAIL against bladder cancer cells. Therefore, further investigations will be required to explain the cellular signaling pathways by which flavones cooperate with TRAIL to induce death. However, only three studies with flavones showed that luteolin and apigenin synergistically induced apoptosis with TRAIL in other human malignant tumor cells [19, 29, 30]. There is no evidence of the cytotoxic effect of TRAIL in combination with flavones on bladder cancer cells. Horinaka et al. reported that luteolin increased TRAIL-induced apoptosis in HeLa cells through up-regulation of death receptor TRAIL-R2 [29]. In another investigation they also showed the enhanced apoptosis-inducing potential of TRAIL in prostate cancer cell line DU145, leukemic cell line Jurkat and colon cancer cell line DLD1. The combined use of apigenin and TRAIL caused activation of caspases and increased expression of TRAIL-R2 [30].

Besides the experimental data, the meta-analysis also confirmed the role of dietary flavonoids and decreased bladder cancer risk in patients with diets rich in fruits and vegetables [5-7]. It has led to an increased emphasis on cancer prevention strategies in which plant flavonoids will be used as dietary components or supplements. Bladder cancer represents a very well suitable disease for chemopreventive intervention. Firstly, its natural history is characterized by frequent recurrences, which need to be reduced or minimized. Secondly, in addition to the role of genetic conditioning, the pathogenesis of bladder cancer also correlates with environmental factors such as cigarette smoking and implying sustained contact of urinary carcinogens with the urothelium. Thirdly, the anatomical location of bladder allows for effective outpatient follow-up examination [6, 7].

The use of naturally occurring dietary agents is becoming increasingly appreciated as an effective strategy of bladder cancer chemoprevention. They could offer a significant reduction in bladder cancer incidence or tumor recurrence and progression after initial treatment and in this way decrease overall healthcare financial burden and improve the quality of life.

## CONCLUSIONS

Epidemiological and preclinical evidence suggest that flavonoids, including flavones, possess cancer chemopreventive properties. It has been indicated that tested flavones (in particular luteolin) enhance the cytotoxic effect of TRAIL on bladder cancer cells. Our findings suggest that dietary flavones supported TRAIL-induced cytotoxicity in TCC cells and might be promising chemopreventive agents in bladder cancer.

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