The dietary flavonol fisetin enhances the apoptosis-inducing potential of TRAIL in prostate cancer cells

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Abstract. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is an endogenous agent that induces apoptosis selectively in cancer cells. Soluble or expressed in immune cells, TRAIL plays an important role in the defense against tumour cells. The resistance of cancer cells to TRAIL immune surveillance is implicated in tumour development. Naturally occurring flavonoids can sensitize TRAIL-resistant cancer cells and augment their apoptotic activity. Fisetin, a dietary flavonol has cancer preventive properties. This study was designed to investigate the effect of fisetin on the TRAIL-induced apoptosis potential in prostate cancer cells. Prostate cancer cell lines represent an ideal model for research in chemoprevention. Cytotoxicity was measured by MTT and LDH assays. Apoptosis was detected using Annexin V-FITC by flow cytometry and fluorescence microscopy. Mitochondrial membrane potential ($\Delta\Psi$m) was evaluated using DePsipher staining by fluorescence microscopy. Death receptor (TRAIL-R1 and TRAIL-R2) expression was analysed by flow cytometry. Inhibition of NF-κB (p65) activation was confirmed with an ELISA-based TransAM NF-κB kit. Caspase-8 and caspase-3 activities were determined by colorimetric protease assays. Our study demonstrates that fisetin sensitizes the TRAIL-resistant androgen-dependent LNCaP and the androgen-independent DU145 and PC3 prostate cancer cells to TRAIL-induced death. Fisetin augmented TRAIL-mediated cytotoxicity and apoptosis in prostate cancer LNCaP cells by engaging the extrinsic (receptor-mediated) and intrinsic (mitochondrial) apoptotic pathways. Fisetin increased the expression of TRAIL-R1 and decreased the activity of NF-κB. Co-treatment of cancer cells with TRAIL and fisetin caused significant activation of caspase-8 and caspase-3 and disruption of $\Delta\Psi$m. Our data indicate the usefulness of fisetin in prostate cancer chemoprevention through enhancement of TRAIL-mediated apoptosis.

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Introduction

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in cancer cells with no toxicity toward normal tissues. TRAIL plays an important role in immune surveillance and in the defence against tumour cells. This death ligand is expressed on the surface of T lymphocytes, natural killer cells, dendritic cells, neutrophils, monocytes, macrophages and can be cleaved into a soluble, secreted form (1-3). TRAIL is a promising antitumour agent capable of killing cancer cells via receptor-mediated (extrinsic) programmed death. TRAIL binds to death receptor TRAIL-R1 (DR4) and/or TRAIL-R2 (DR5), which promotes the recruitment of the adaptor molecule Fas-associated death domain (FADD) to form the death inducing signalling complex (DISC) and activate caspase-8. Crosstalk exists between the extrinsic and intrinsic (mitochondrial-dependent) apoptosis pathways. Activation of caspase-8 directly leads to the activation of caspase-3, causing cell death, and simultaneously, BH3-interacting domain death agonist (Bid) cleavage along with cytochrome c release and mitochondrial membrane potential disruption (4,5). However, some tumour cells are resistant to TRAIL-induced cytotoxicity. Failure to undergo apoptosis has been implicated in the resistance of cancer cells to TRAIL surveillance and therefore in tumour development. The expression of the death receptors and proapoptotic or antiapoptotic proteins in cancer cells is involved in TRAIL-resistance (1-8). Studies of our group as well as of other researchers have shown that TRAIL-resistant cancer cells can be sensitized to TRAIL-mediated apoptosis by flavonoids (9-16).

Flavonoids, a family of polyphenolic compounds, are commonly found in fruits and vegetables. They are subdivided into several groups, including flavonols, flavonones, flavones, flavanols, isoflavones and anthocyanidins. As natural products, flavonoids are regarded as safe and easily obtainable in routine diet, a fact that makes them ideal cancer chemopreventive agents (3,17-19). Fisetin (3,3',4',7-tertahydroxyflavone) is a flavonol present in apple, strawberry, grape, kiwi fruit, persimmon, cucumber and onion. The structure of the

compound is presented in Fig. 1. Fisetin induces apoptosis and cell cycle arrest in prostate cancer LNCaP, DU145 and PC3 cells, with minimal effects on normal prostate epithelial cells (20-23). The treatment of athymic nude mice with this flavonol results in inhibition of CWR22Rv1-based prostate tumour xenograft growth (24).

Chemopreventive intervention using naturally occurring dietary agents is an attractive option in prostate cancer because of its incidence, prevalence, and disease-related morbidity and mortality (20,25,26). Epidemiological studies have demonstrated an inverse association between flavonoid intake and prostate cancer risk (17,27). Flavonoids exert their chemopreventive activity through the modulation of apoptotic signalling pathways (17). We show here that fisetin markedly augments TRAIL-mediated apoptosis in prostate cancer cells. This dietary flavonol sensitizes androgen-dependent LNCaP cells and androgen-independent DU145, PC3 cells to TRAIL-induced apoptosis.

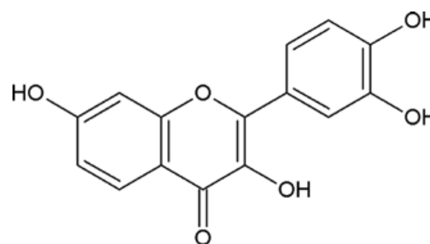

Figure 1. Chemical structure of fisetin.

Materials and methods

Prostate cancer cell culture. Human androgen-dependent LNCaP, androgen-independent DU145 and androgen-independent PC3 prostate cancer cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The LNCaP and DU145 cells were maintained in RPMI-1640 medium. The PC3 cells were grown in RPMI-1640 with the addition of Ham's medium. The media for prostate cell cultures were supplemented with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 (27,28). All reagents for cell culture were purchased from PAA, The Cell Culture Company (Pasching, Austria).

Reagents. Fisetin was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). The flavonoid was dissolved in DMSO (dimethyl sulfoxide) to obtain the working concentrations. Soluble recombinant human TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). The human recombinant chimeric proteins TRAIL-R1/Fc and TRAIL-R2/Fc, the general caspase inhibitor Z-VAD-FMK, the caspase-8 inhibitor Z-IETD-FMK, and the caspase-3 inhibitor Z-DEVD-FMK were obtained from R&D Systems (Minneapolis, MN, USA).

Cytotoxicity assay. Cytotoxicity was measured by the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (29,30). The MTT assay is based on the cleavage of the tetrazolium salt MTT by viable cells to a blue formazan dye. LNCaP cells ($2x10^5$/ml), DU145 cells ($1x10^5$/ml) and PC3 ($1x10^5$/ml) cells were seeded in a 96-well plate for 24 h before the experiments. Then the cells were incubated with TRAIL (20-200 ng/ml) and/or fisetin (10-50 μM). After 24 h, 20 μl of MTT solution (5 mg/ml) was added to each well for 4 h. The resulting blue formazan crystals were dissolved in DMSO. These reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Controls included native cells and medium alone. The spectrophotometric absorbance at 550 nm wavelength was measured using a microplate reader (ELx 800) (Bio-Tek Instruments, Inc., Winooski, VT, USA). The percent cytotoxicity was calculated by the following formula: percent cytotoxicity (cell death) = (1- [absorbance of experimental wells/absorbance of control wells]) x 100%.

Lactate dehydrogenase release assay. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon membrane damage in necrotic cells. LDH activity was measured using a cytotoxicity assay kit (Roche Diagnostics GmbH, Mannheim, Germany) (30,31). Prostate cancer cells were treated with TRAIL (20-200 ng/ml) and/or fisetin (10-50 μM) for the indicated period of time. LDH released in culture supernatants was detected with a coupled enzymatic assay, resulting in the conversion of a tetrazolium salt into a red formazan product. The spectrophotometric absorbance at 490 nm was measured using a microplate reader. Maximal release was obtained after treating control cells with 1% Triton X-100 (Sigma Chemical Co.) for 10 min at room temperature. The necrotic percentage was expressed using the following formula: (sample value/maximal release) x 100%.

Detection of apoptosis by flow cytometry. Apoptosis was determined by flow cytometry using the Apoptest-FITC kit with Annexin V (Dako, Glostrup, Denmark). LNCaP cells ($2x10^5$/ml), DU145 cells ($1x10^5$/ml) and PC3 cells ($1x10^5$/ml) were seeded in 24-well plates for 24 h and then exposed to TRAIL (20-100 ng/ml) and/or fisetin (10-50 μM) for 24 h. After this incubation, the cancer cells were washed twice with phosphate-buffered saline solution (PBS) and resuspended in 1 ml of binding buffer. The cell suspension (500 μl) was then incubated with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) for 10 min at room temperature in the dark. The population of Annexin V-positive cells was evaluated by flow cytometry (BD FACScan, Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) (31,32).

Detection of apoptosis by fluorescence microscopy. Apoptotic cells were quantified using the fluorescence microscopy method of the Apoptotic and Necrotic and Healthy Cells Quantification kit from Biotium, Inc. (Hayward, CA, USA) (15,33). LNCaP cells ($2x10^5$/ml), DU145 cells ($1x10^5$/ml) and PC3 cells ($1x10^5$/ml) were seeded in 24-well plates for 24 h before the experiments. TRAIL (100 ng/ml) and/or fisetin (50 μM) were added to the cancer cells, and 24 h later, the cells were washed with PBS and detached from the cell culture wells with trypsin. Next, the cells were centrifuged to discard the supernatant, washed with PBS and resuspended in binding buffer (100 μl/sample). A combination of 5 μl of Annexin V-FITC, 5 μl of ethidium homodimer III and 5 μl of Hoechst 33342 solution was added

to each tube. The samples were incubated at room temperature for 15 min in the dark. After staining, the cancer cells were washed with binding buffer, placed on a glass slide and covered with a glass coverslip. The stained cells were observed under an IX51 fluorescence inverted microscope (Olympus, Tokyo, Japan) using filter sets for FITC, TRITC and DAPI. The cells were counted, and the number of apoptotic cells was expressed as a percentage of the total cells.

Flow cytometric analysis of death receptor expression on the cell surface. The cell surface expression of death receptors TRAIL-R1 and TRAIL-R2 on LNCaP cells was determined by flow cytometry (BD FACSCanto, Becton-Dickinson Immunocytometry Systems). LNCaP cells ($2x10^5$/ml) were seeded in 24-well plates for 24 h and then exposed to fisetin (50 μM) for 24 h. Cells were then harvested using trypsin and ethylenediaminetetraacetic acid (EDTA), washed twice in PBS and resuspended in PBS containing 0.5% bovine serum albumin (BSA). Cells were incubated with 10 μl phycoerythrin-conjugated anti-TRAIL-R1 or anti-TRAIL-R2 monoclonal antibodies (R&D Systems) at 4˚C for 45 min. After staining, the cancer cells were washed with PBS and finally analysed by flow cytometry (35-37). The control consisted of cells in a separate tube treated with phycoerythrin-labelled mouse IgG_1 or mouse IgG_{2B} (R&D Systems).

Caspase activity assays. Caspase-3 and caspase-8 activities were assessed by colorimetric protease assay kits (R&D Systems). The tests are based on the spectrophotometric detection of the chromophore p-nitroanaline (pNA) after cleavage from the caspase substrate (a caspase-specific peptide conjugated to pNA). LNCaP cells ($1x10^6$/ml) were seeded in Petri dishes 24 h before the experiments. TRAIL (100 ng/ml) and/or fisetin (50 μM) were added to the cancer cells, and 24 h later, the cells were washed with PBS and detached from the cell culture wells by trypsin. The cells were centrifuged to discard the supernatant and treated with lysis buffer. The cell lysates were tested for protease activity by the addition of a labelled caspase substrate, DEVD-pNA for caspase-3 activity and IETD-pNA for caspase-8 activity. pNA absorbance was quantified using a spectrophotometer V-630 (Jasco International Co., Tokyo, Japan) at a wavelength of 405 nm (38).

Evaluation of the mitochondrial membrane potential by DePsipher. The DePsipher kit (R&D Systems) was used to measure the mitochondrial membrane potential under a fluorescence microscope (34,35). LNCaP cells ($2x10^5$/ml) were seeded in a 24-well plate 24 h prior to the experiments. TRAIL (100 ng/ml) and/or fisetin (50 μM) were added, and 24 h later, the cells were washed with PBS and detached from the cell culture wells with trypsin. The cells were incubated in the dark with DePsipher (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl carbocyanin iodide) solution at a concentration of 5 μg/ml for 30 min at 37˚C, washed with reaction buffer with stabiliser, placed on a glass slide and covered with a glass coverslip. The stained cells were observed under a fluorescence inverted microscope using filter sets for FITC and TRITC. DePsipher visualizes the potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from red (590 nm) to green (530 nm).

The activity of NF-κB. The NF-κB activity in nuclear extracts was measured using the ELISA-based TransAM NF-κB kit (Active Motif Europe, Rixensart, Belgium). LNCaP cells ($1x10^6$/ml) were seeded in Petri dishes 24 h before the experiments. Fisetin (50 μM) with or without TRAIL (100 ng/ml) was added to the cancer cells, and 24 h later, the cells were washed with PBS and detached from the cell culture wells by trypsin treatment. The commercially available Nuclear Extract kit was obtained from Active Motif Europe for the preparation of LNCaP cell nuclear extracts. The TransAM NF-assay for NF-κB (p65) activity was performed according to the vendor's protocol (35,37). NF-κB DNA-binding activity was assessed using the ELISA kit for the transcription factor p65. Oligonucleotides containing the NF-κB consensus site (5'-GGGACTTCC-3') were immobilised on a 96-well plate. The active forms of NF-κB in the nuclear extracts were bound to the oligonucleotides on the plate and detected colourimetrically by spectrophotometry at an absorbance of 450 nm and with a reference wavelength of 650 nm. The detection limit for the TransAM NF-κB kit is <0.4 ng/ml purified p65.

Statistical analysis. The results are expressed as the mean ±SD obtained from three independent experiments performed in quadruplicate (n=12) or duplicate (n=6). The statistical significance was evaluated using the Bartlett χ^2 test followed by analysis of variance (ANOVA) and post-hoc test. The expression of death receptors was assessed using the Levene test followed by the Mann-Whitney U-test. A P-value <0.05 was considered significant.

Results

Cytotoxic and apoptotic effects of TRAIL in combination with fisetin in the androgen-dependent LNCaP prostate cancer cells. The cytotoxic effect of TRAIL at the concentrations of 20-100 ng/ml after 24-h incubation ranged from 3.8±1.4% with the lowest concentration to 14.2±1.2% cell death with the highest concentration. The death ligand at the same concentrations induced apoptosis ranging from 7.5±0.9 to 16.8±0.9% in prostate cancer cells. TRAIL concentrations higher than 100 ng/ml resulted in no significant increase in apoptotic activity. LNCaP cells were resistant to TRAIL-mediated cytotoxicity and apoptosis. We next investigated the cytotoxic and apoptotic effects of TRAIL in combination with fisetin on LNCaP cells. After 24 h of co-treatment of cancer cells with TRAIL at concentrations of 20-100 ng/ml and flavonol at concentrations of 10-50 μM the cytotoxicity ranged from 25.2±1.0% (10 μM fisetin plus 20 ng/ml TRAIL) to 81.2±1.6% (50 μM fisetin plus 100 ng/ml TRAIL). The cytotoxicity measured by the MTT assay is shown in Fig. 2A. We found that fisetin synergized with TRAIL to induce apoptosis in cancer cells. The percentage of apoptotic cells stained with Annexin V-FITC detected by flow cytometry after a 24-h exposure to TRAIL at 50-100 ng/ml in combination with 20-50 μM fisetin was elevated to 61.6±1.0-80.6±1.1% (Fig. 2B). Fisetin sensitized TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis. The necrotic cell death percentage of LNCaP cells examined by the Apoptest-FITC and the LDH assays was nearly zero. The Annexin V-FITC staining visualised by fluorescence microscopy supports the

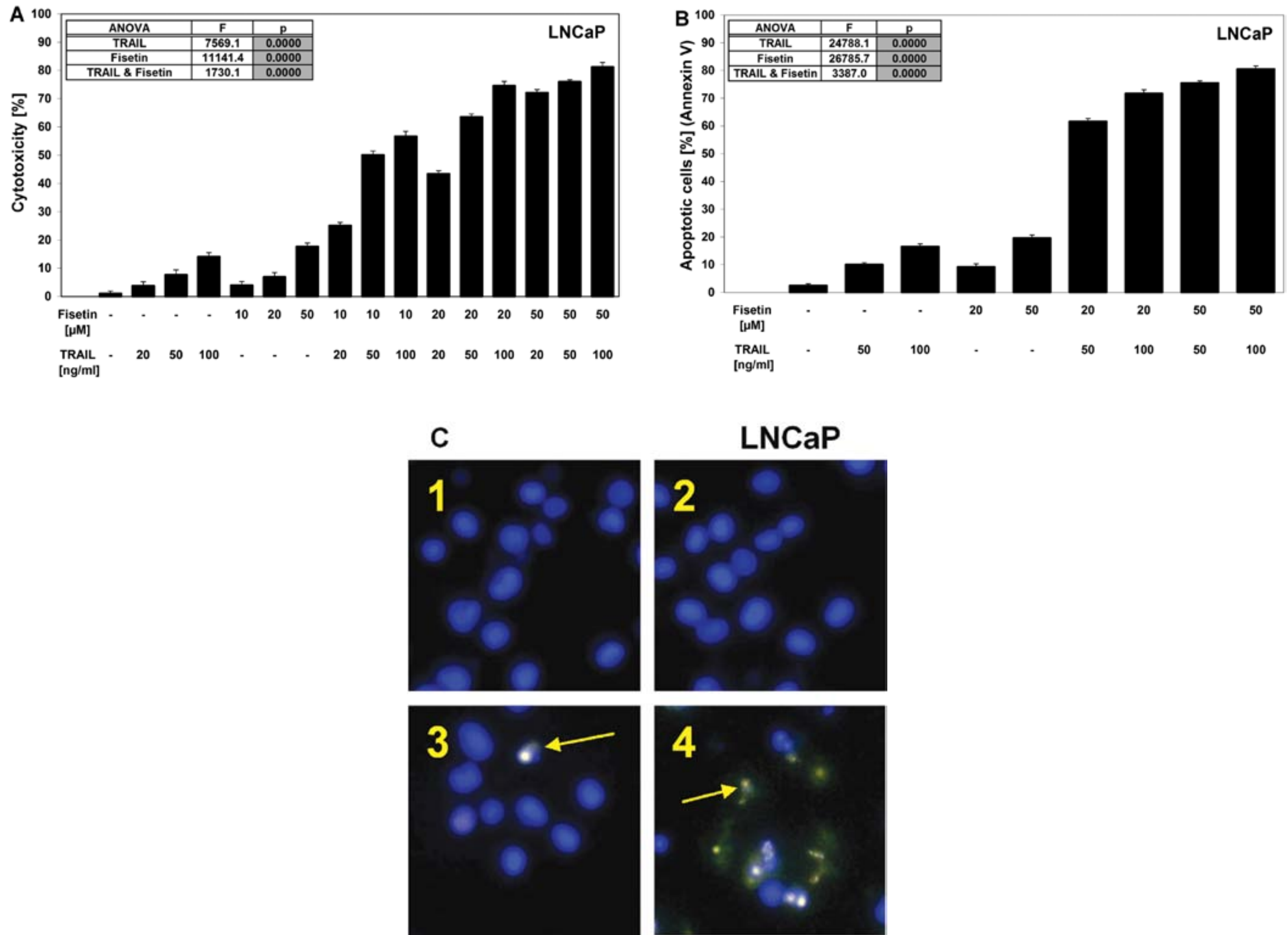

Figure 2. Cytotoxic and apoptotic effects of TRAIL in combination with fisetin in the androgen-dependent prostate cancer cells. (A) The cytotoxic activity of TRAIL in combination with fisetin in LNCaP cells. Cells were incubated for 24 h with TRAIL at concentrations of 20-100 ng/ml and/or fisetin at concentrations of 10-50 μM. The percentage of cell death was measured using the MTT cytotoxicity assay. The values represent the mean ± SD of three independent experiments performed in quadruplicate (n=12) (P<0.0001 TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). (B) TRAIL-induced apoptosis in combination with fisetin in LNCaP cells. Cells were incubated for 24 h with TRAIL at concentrations of 50-100 ng/ml and/or fisetin at concentrations of 20-50 μM. Apoptotic cell death was detected by flow cytometry using Annexin V-FITC staining. The values represent the mean ± SD of three independent experiments performed in duplicate (n=6) (P<0.0001 for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). (C) TRAIL-induced apoptosis in combination with fisetin in LNCaP cells: (1) control cells, (2) cells incubated with 100 ng/ml TRAIL, (3) cells incubated with 50 μM fisetin and (4) cells incubated with both 100 ng/ml TRAIL and 50 μM fisetin. Apoptotic cell death was detected and visualised by fluorescence microscopy using Annexin V-FITC staining. Healthy cells (stained with Hoechst 33342) emitted blue fluorescence, and apoptotic cells (stained with Hoechst 33342 and Annexin V-FITC) emitted green and blue fluorescence (indicated by arrows).

augmentation of the apoptotic activity of TRAIL by fisetin against LNCaP cells (Fig. 2C).

Cytotoxic and apoptotic effects of TRAIL in combination with fisetin in the androgen-independent DU145 and PC3 prostate cancer cells. The cells were incubated with 100 ng/ml TRAIL for 24 h. The cytotoxicity of TRAIL was 5.5±1.4% in the DU145 cells and 4.1±1.0% in the PC3 cells. Exposure of DU145 and PC3 cells to 100 ng/ml TRAIL resulted in the presence of 7.5±0.6% and 7.1±0.7% of apoptotic cells, respectively. Both androgen-independent prostate cancer cell lines were resistant to TRAIL. The co-treatment of cancer cells with 100 ng/ml TRAIL and 50 μM fisetin increased the percentage of cell death to 58.1±2.2% for DU145 cells and to 50.1±1.1% for PC3 cells (Fig. 3A and B). TRAIL in combination with 50 μM fisetin induced 55.3±1.2% apoptosis in DU145 cells and 50.8±1.1% in PC3 cells (Fig. 3C and D). Annexin V-staining visualised by fluorescence microscopy was used to demonstrate these effects (Fig. 3E and F). The necrotic cell death percentage of cancer cells examined by the Apoptest-FITC and the LDH assays was nearly zero. Fisetin enhanced the apoptosis-inducing potential of TRAIL and sensitized the prostate cancer DU145 and PC3 cells.

Effects of fisetin on death receptor expression in LNCaP cells. The expression of death receptors on the cell surface is critical for TRAIL-mediated apoptosis. We therefore, analysed the expression of TRAIL-R1 and TRAIL-R2 in LNCaP cells after a 24-h treatment with 50 μM fisetin by flow cytometry (Fig. 4). Fisetin significantly increased the expression of TRAIL-R1 in prostate cancer cells, but TRAIL-R2 expression was unaltered. Fisetin enhanced the apoptotic effect of TRAIL through the intrinsic apoptosis pathway. To confirm that the apoptosis induced by co-treatment with TRAIL and fisetin

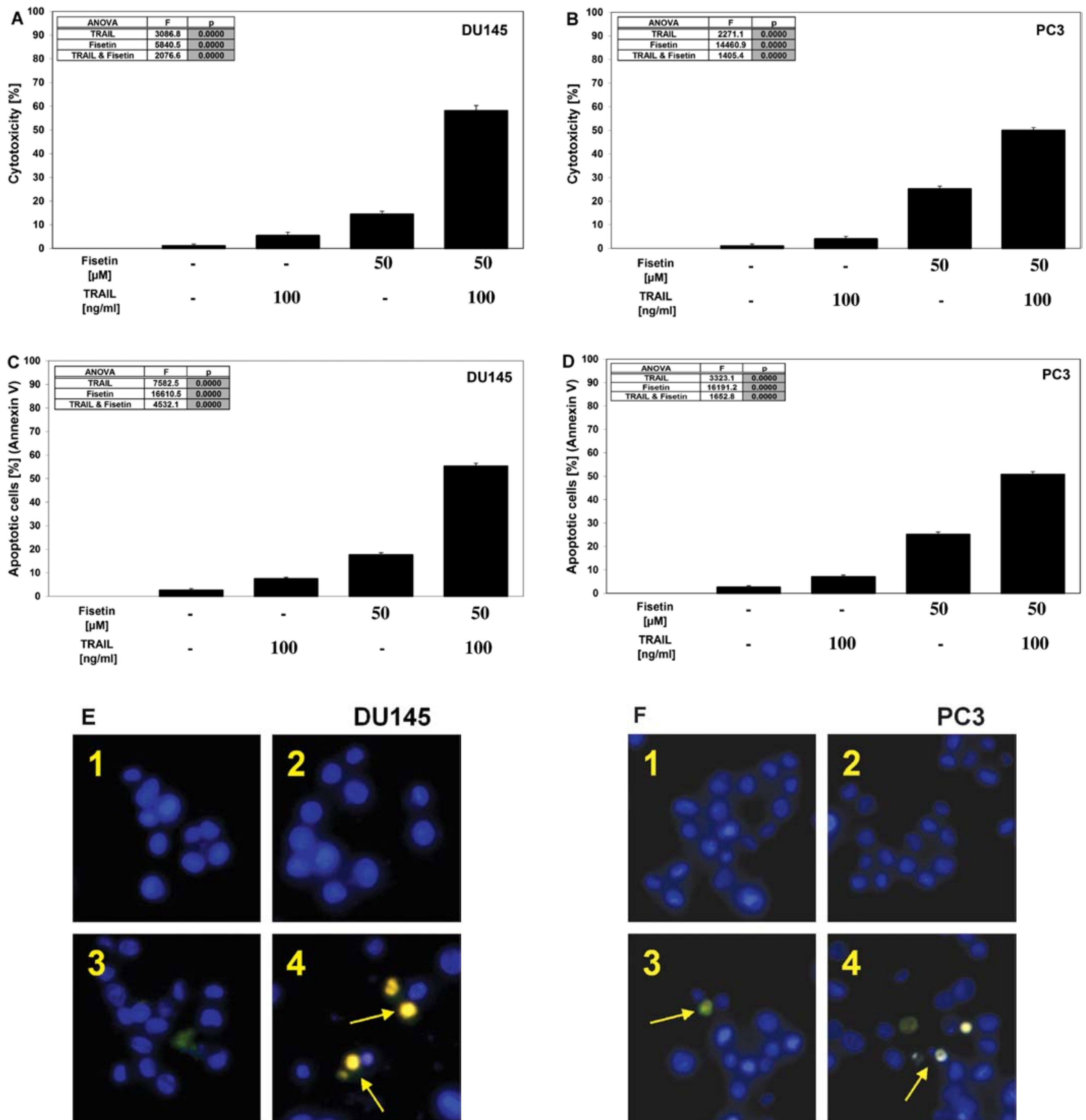

Figure 3. Cytotoxic and apoptotic effects of TRAIL in combination with fisetin in androgen-independent prostate cancer cells. Cells were incubated for 24 h with TRAIL at a concentration of 100 ng/ml and/or fisetin at concentration of 50 μM. The cytotoxic activity of TRAIL in combination with fisetin in (A) DU145 cells and (B) PC3 cells. The percentage of cell death was measured using the MTT cytotoxicity assay. The values represent the mean ± SD of three independent experiments performed in quadruplicate (n=12) ($P< 0.0001$ for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). TRAIL-induced apoptosis in combination with fisetin in (C) DU145 and (D) PC3 cells. Apoptotic cell death was detected by flow cytometry using Annexin V-FITC staining. The values represent the mean ± SD of three independent experiments performed in duplicate (n=6) ($P<0.0001$ for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). TRAIL-induced apoptosis in combination with fisetin in (E) DU145 cells and (F) PC3 cells: (1) control cells, (2) cells incubated with 100 ng/ml TRAIL, (3) cells incubated with 50 μM fisetin, and (4) cells incubated with both 100 ng ml TRAIL and 50 μM fisetin. Apoptotic cell death was detected and visualised by fluorescence microscopy using Annexin V-FITC staining. Healthy cells (stained with Hoechst 33342) emitted blue fluorescence, and apoptotic cells (stained with Hoechst 33342 and Annexin V-FITC) emitted green and blue fluorescence (indicated by arrows).

was mediated through TRAIL-R1, we used the TRAIL-R1/Fc chimeric protein, which has a dominant negative function against TRAIL-R1. The protein efficiently blocked apoptosis caused by the co-treatment of TRAIL and fisetin.

Effects of TRAIL and fisetin on caspase-8 and caspase-3 activities in LNCaP cells. The cells were treated with 100 ng/ml TRAIL and/or 50 μM fisetin for the indicated period of time. The stimulation of death receptors induces DISC formation,

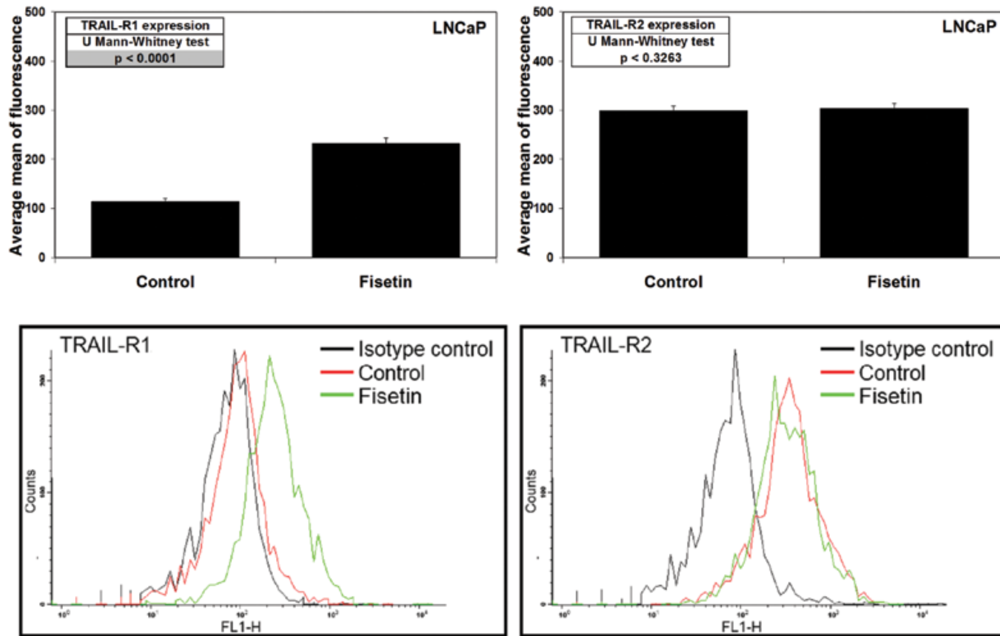

Figure 4. Effects of fisetin on death receptor expression in LNCaP prostate cancer cells. Cells were incubated for 24 h with 50 μM fisetin. The surface expression of TRAIL-R1 and TRAIL-R2 on LNCaP cells was measured by flow cytometry. Representative histograms and the average mean fluorescence from three independent experiments were performed in duplicate n=6. The values represent mean ± SD (P<0.0001 fisetin compared to control with the Mann-Whitney U test).

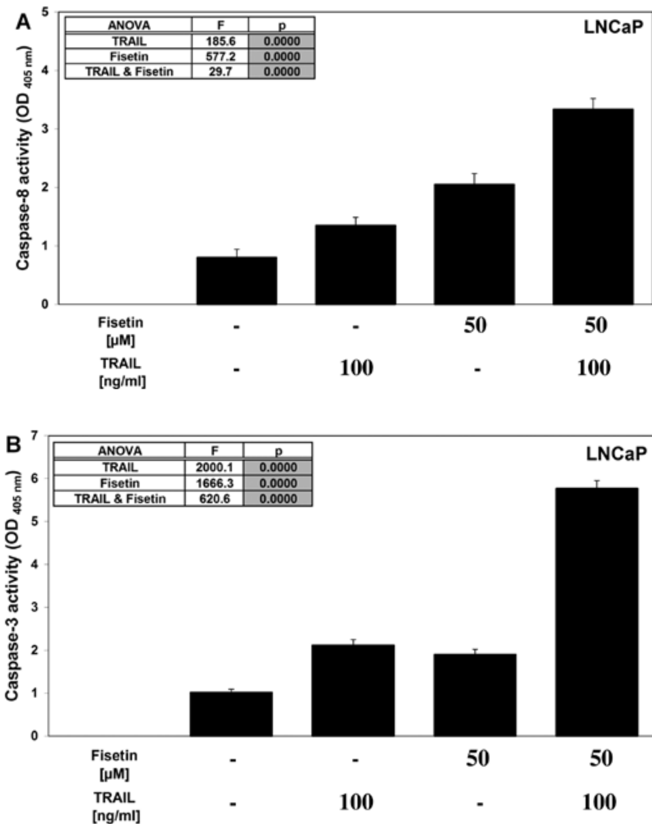

Figure 5. Effects of TRAIL in combination with fisetin on caspase activities in LNCaP prostate cancer cells. Assessment of intracellular (A) caspase-8 and (B) caspase-3 activity in LNCaP treated with 100 ng/ml TRAIL and/or 50 μM fisetin for 24 h. Caspase activities were measured by colorimetric protease assays based on the spectrophotometric detection of the chromophore p-nitroanaline (pNa) after cleavage from the labelled caspase substrates. The values represent the mean ± SD of three independent experiments performed in duplicate (n=6) (P<0.0001 for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA).

which results in the recruitment and activation of caspase-8. TRAIL and fisetin alone activated caspase-8 in cancer cells. Simultaneous incubation of LNCaP cells with TRAIL and fisetin markedly increased caspase-8 activity (Fig. 5A). Caspase-3 is an effector caspase that plays a central role in apoptosis. Co-treatment of cancer cells with TRAIL and fisetin significantly enhanced caspase-3 activity, compared to treatment with TRAIL or fisetin alone (Fig. 5B). Use of the pan-caspase inhibitor Z-VAD-FMK, the caspase-8 inhibitor Z-IETD-FMK or the caspase-3 inhibitor Z-DEVD-FMK completely blocked the subsequent cell death induced by TRAIL in combination with fisetin. These findings demonstrate that fisetin is able to promote TRAIL-mediated apoptosis through a caspase cascade.

Effects of TRAIL and fisetin on the mitochondrial membrane potential (ΔΨm) in LNCaP cells. Mitochondrial membrane depolarization is one of the first intracellular changes following the onset of apoptosis. We therefore, determined whether fisetin sensitizes cancer cells to TRAIL-induced mitochondrial dysfunction. Treatment of LNCaP cells with 100 ng/ml TRAIL or 50 μM fisetin alone resulted in a small effect on the ΔΨm (12.4±0.9 and 17.8±1.0%, respectively). The combination of TRAIL and fisetin enhanced ΔΨm loss in a large percentage of cancer cells (75.6±1.1%) and induced a significant disruption of the ΔΨm (Fig. 6). These results suggest that the extrinsic apoptosis pathway is involved in LNCaP cells treated with TRAIL and fisetin.

Effects of fisetin and TRAIL on NF-κB activity in LNCaP cells. We examined the effects of fisetin and/or TRAIL on NF-κB activation in cancer cells (Fig. 7). We evaluated the binding activity of the p65 subunit in nuclear extracts with the

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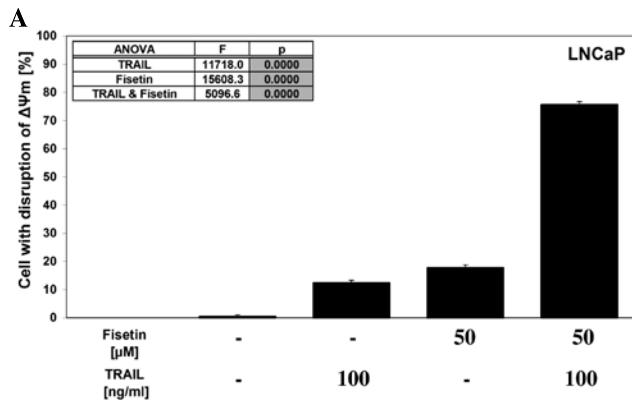

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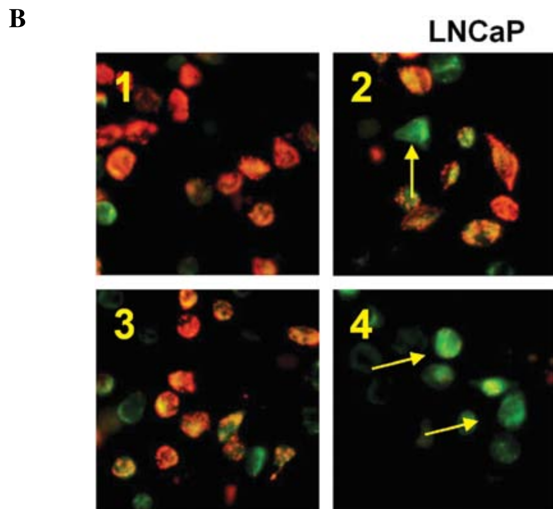

Figure 6. Effects of TRAIL in combination with fisetin on the mitochondrial membrane potential (ΔΨm) in LNCaP prostate cancer cells. Cells were incubated for 24 h with 100 ng/ml TRAIL and/or 50 μM fisetin. The values represent the mean ± SD of three independent experiments performed in duplicate (n=6) (P< 0.001 for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). (A) TRAIL in combination with fisetin induced loss of ΔΨm in LNCaP cells. (B) Disruption of ΔΨm in cancer cells was assessed by fluorescence microscopy of DePsipher staining: (1) control cells, (2) cells incubated with 100 ng/ml TRAIL, (3) cells incubated with 50 μM fisetin, and (4) cells incubated with both 100 ng/ml TRAIL and 50 μM fisetin. Red fluorescence is emitted from the red aggregates of DePsipher, which are formed within mitochondria of healthy cells. Green fluorescence reveals the monomeric form of the DePsipher molecule, which appears in the cytosol after mitochondrial membrane depolarisation (indicated by arrows).

ELISA-based TransAM NF-κB kit. Fisetin decreased the activity of NF-κB compared with the control. In contrast, TRAIL induced the activation of NF-κB in LNCaP cells. Co-treatment of fisetin with TRAIL significantly decreased the NF-κB activity. The tested flavonol blocked the NF-κB activation induced by TRAIL in prostate cancer cells and in this way overcame the resistance to TRAIL.

Discussion

The use of dietary polyphenols is becoming increasingly appreciated as an effective strategy in prostate cancer prevention. Recently, there has been major interest in the development of compounds of natural origin with chemopreventive activities (20,25). Flavonoids isolated from fruits, vegetables, green and black tea, herbs, spices, propolis, beer and red wine are

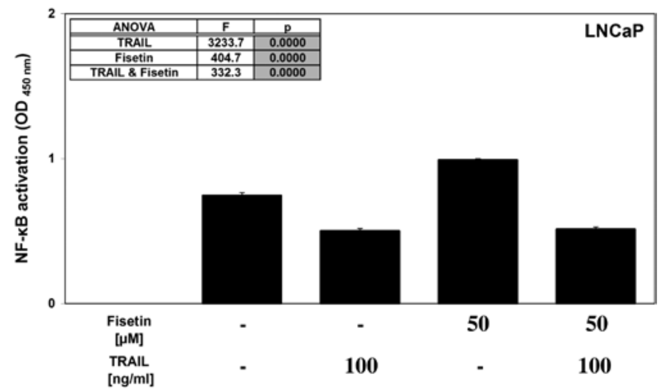

Figure 7. Effects of fisetin and TRAIL on NF-κB activity in LNCaP prostate cancer cells. The cancer cells were incubated for 24 h with 50 μM fisetin and/or 100 ng/ml TRAIL. The values represent the mean ± SD of three independent experiments performed in duplicate (n=6) (P<0.0001 for TRAIL + fisetin compared to TRAIL or fisetin alone in ANOVA). The effects of fisetin and/or TRAIL on the NF-κB (p65) binding activity in nuclear extracts from LNCaP cells were measured using the ELISA-based TransAM NF-κB assay.

extensively researched for their health promoting potential. Epidemiological and preclinical findings confirm the anticancer and chemopreventive properties of flavonoids (3,17-20).

TRAIL is an important component of the immune defence that causes apoptosis selectively in cancer cells (1). Dysregulation of apoptotic pathways is significant in the initiation and progression of prostate cancer (25,26,39-41). LNCaP, DU145 and PC3 prostate cancer cells are resistant to TRAIL-mediated apoptosis (5,11,28). Several *in vitro* tests suggest that the chemopreventive advantages of dietary flavonoids are associated with the enhancement of TRAIL-induced death in cancer cells (3,42,43). Although the majority of prostate cancer cell lines are resistant to TRAIL-mediated apoptosis, TRAIL in combination with flavonoids results in the synergistic induction of cell death (9-14). Studies on the intracellular mechanism of TRAIL-mediated apoptosis may help overcome TRAIL-resistance and develop flavonoids-based approaches to prostate cancer prevention.

Apoptosis can be triggered by two major mechanisms: the intrinsic pathway involving mitochondrial dysfunction, and the extrinsic pathway stimulated by death receptors located on the cell membrane (1). The death receptors are essential for receptor-depending apoptosis in cancer (6,7). We observed that fisetin enhanced TRAIL-apoptosis through up-regulation of TRAIL-R1 expression. Siddqui *et al* also showed that the green tea flavonoid epigallocathechin-3-gallate (EGCG) increases the expression of TRAIL-R1, thereby sensitizing LNCaP cells to TRAIL-mediated apoptosis (12). In prevalent cases, flavonoids can reverse TRAIL-resistance of prostate cancer cells, accompanied by the up-regulation of TRAIL-R2. Apigenin, baicalein, quercetin and biochanin-A influence TRAIL-R2 expression in prostate cancer cells (9,10,13,37). Caspases comprise the central machinery in apoptosis. TRAIL-mediated programmed cell death is mainly executed by the extrinsic death receptor pathway, involving caspase-8 as the initiator and caspase-3 as the executor caspase. We observed that TRAIL or fisetin alone activated caspase-8 and caspase-3 in LNCaP cells, whereas co-treatment with TRAIL

and fisetin resulted in significant activation of both caspases in prostate cancer cells. Caspases 8, 9 and 3 are activated by TRAIL in combination with EGCG in LNCaP cells or with quercetin in DU145 cells (11-13). These data provide further evidence that sensitization of prostate cancer cells to TRAIL by fisetin is achieved through a receptor- and caspase-dependent pathway. Our previous studies on prostate cancer cells confirmed the role of mitochondrial dysfunction caused by isoflavones and chalcones in TRAIL-mediated apoptotic pathways (34,37,44). Therefore, we analysed the $\Delta\Psi m$ in LNCaP cells after incubation with TRAIL and/or fisetin. TRAIL and fisetin co-treatment affected the extrinsic pathway in prostate cancer cells via significant reduction of $\Delta\Psi m$ compared to TRAIL or fisetin alone.

Constitutively high expression of NF-κB occurs frequently during cancer initiation and progression. De-regulated activation of NF-κB promotes survival of tumour cells and resistance to apoptosis. The activation of NF-κB in prostate cancer cells is associated with resistance to TRAIL-mediated death (45). Inhibition of NF-κB activation by fisetin in LNCaP cells augmented the apoptotic effect of TRAIL. Our findings confirm that the down-regulation of NF-κB sensitizes prostate cancer cells to TRAIL *in vitro*.

Overcoming TRAIL-resistance by fisetin exposure in LNCaP cells was due to the up-regulation of TRAIL-R1, activation of caspase-8 and caspase-3, loss of $\Delta\Psi m$ and down-regulation of NF-κB. We showed for the first time that fisetin sensitizes androgen-dependent LNCaP cells or androgen-independent DU145 and PC3 cells to TRAIL-induced apoptosis. Interestingly, in androgen-independent prostate cancer cell lines the cytotoxic and apoptotic effects of TRAIL in combination with fisetin are lowest compared to androgen-dependent LNCaP cells. The results suggest that fisetin could be a promising chemopreventive agent for early-stage prostate cancer.

The apoptotic activity of fisetin alone has been established previously (46-48). Lim and Park demonstrated that fisetin increases the expression of TRAIL-R2, the cleavage of caspases 8, 9, 7, 3 and the permeability of the mitochondrial membrane with cytochrome c release in colon cancer HCT116 cells (47). Khan *et al* showed that fisetin activates caspases 8, 9, and 3, and causes mitochondrial release of cytochrome c into the cytosol in prostate cancer LNCaP cells (21). Chien *et al* (22) and Li *et al* (48) reported the suppression of NF-κB activation in bladder cancer T24 cells and prostate cancer PC3 cells incubated with fisetin. The data provide strong evidence, that this flavonol exhibits anticancer and chemopreventive properties. Our *in vitro* study indicates the significance of fisetin in prostate cancer chemoprevention strategies through enhancing the apoptosis inducing potential of TRAIL.

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