In vitro antiproliferative and antiangiogenic effects of Flavin7®

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Short title: Anticancer effect of Flavin7

Summary

Flavin7 (F7) is a nutritional supplement often taken by cancer patients in Central Europe during chemo- and radiation therapy. In this study, investigation of the antiproliferative and antiangiogenic activities of this supplement were performed.

Flavin7 showed antiproliferative activity in Jurkat as well as in HeLa cells. It significantly reduced the growth of both cancer cell lines from 1/5 to 1/50 dilutions ($p<0.001$and 0.01, respectively). In F7-treated Jurkat cells we found a significant increase in the fraction of cells with sub-G_0/G_1 DNA content, which is considered to be a marker of apoptotic cell death. Apoptosis was also confirmed by annexin V staining and DNA fragmentation. Furthermore, F7 at 1/100 and 1/250 dilution inhibited endothelial cell migration and capillary tube formation what indicates its potential antiangiogenic properties. Flavin7 also inhibited the activity of matrix metalloproteinases (MMPs), preferentialy MMP-9, at dilutions of 1/10 to 1/250.

Our data suggest that F7 possesses marked antiproliferative and antiangiogenic properties *in vitro*. Further research is needed to elucidate also its *in vivo* activities.

Key words: antiproliferative – antiangiogenic – polyphenols – Flavin7

Introduction

Cancer is a major disease at a worldwide level accounting for more than 7 million deaths/year. Unfortunately, current therapeutic modalities for advanced disease are of limited effectivenes. Alternative options for patients with malignancies include herbal treatments that have been used for many years throughout the world (Risberg *et al.* 1998). A wide variety of plant products is available in natural health products stores as dietary supplements for cancer patients.

Flavin7® (F7) is a nutritional supplement taken by cancer patients during radiation therapy or chemotherapy. It is popular mainly in Central Europe, but it is also marketed in Canada. It has been officially endorsed by the Hungarian Cancer Society as an effective product for enhancing the health of those coping with chemotherapy treatments (http://www.flavin-7.com/hcs.php).

There are no reported studies in the literature of either *in vitro* or *in vivo* studies on the antiproliferative effects of F7 on cancer cells. However, flavonoids and resveratrol, the main active compounds in F7, display a remarkable spectrum of biochemical activities including those that might be able to influence processes that are dysregulated during cancer development. These include *e.g.* antioxidant activities (Cavallaro *et al*., 2003), the scavenging effect on activated carcinogens and mutagens (Ioannides and Yoxall 2003), the action on proteins that control cell cycle progression (Li *et al*. 2005) and gene expression (Gerritsen 1998). Moreover, it was found that some polyphenolic agents possess also antiangiogenic properties (Oak *et al.* 2005).

The purpose of this study was to assess the antiproliferative as well as antiangiogenic properties of F7 *in vitro.*

Material and Methods

Reagents and drugs

MTT, 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was from Sigma-Aldrich Chemie (Steinheim, Germany), Cycle TEST™ PLUS DNA Reagent Kit, annexin V-FITC and propidium iodide were purchased from Becton Dickinson Biosciences (-BDB-, USA). Collagenase II was purchased from Invitrogen (USA). Flavin7® was a gift from Vita Crystal Slovakia Ltd (Slovak Republic).

Tumor cell lines

Jurkat cells (human acute T-lymphoblastic leukemia) and HeLa cells (human cervical cancer) were kindly provided by Dr. M. Hajduch (Olomouc, Czech Republic). The cells were routinely maintained in RPMI 1640 medium with Glutamax-I supplemented with 10% foetal calf serum, penicillin (100 $IU.ml^{-1}$) and streptomycin (100 $\mu g.ml^{-1}$) (all from Invitrogen, USA), in the atmosphere of 5% CO_2 in humidified air at 37°C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

Cytotoxicity assay

The cytotoxic effect of F7 was studied by using colorimetric microculture assay with the MTT end-point (Mosmann 1983). Briefly, $8x10^4$ cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing the tested chemicals at final dilutions of 1/5 to 1/500. After 72 hours of incubation, 10 µl of MTT (5 $mg.ml^{-1}$) (Sigma, Germany) were added in each well. After an additional 4 h, during which insoluble formazan was produced, 100 µl of 10% sodium dodecylsulphate were added in each well and another 12 h were allowed for the formazan to be dissolved. The absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, UK). The absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control.

Cell cycle analysis

The cell cycle distribution in cells treated with F7 was analyzed using Cycle TEST^{TM} PLUS DNA Reagent Kit (BDB). Briefly, $5x10^5$ Jurkat cells were treated with F7 for 24 and 48 hours. After treatment, cells were harvested, washed thrice in citrate buffer and stained according to the manufacturer's instructions. Data acquisition was performed within one hour after staining in a FACS Vantage SE flow cytometer using CellQuest Pro software (both from BDB), information being stored for $5x10^4$ events per sample. The data were analyzed using Win MDI software. Percentages of cells corresponding to G_0/G_1, S and G_2/M cell cycle phases were calculated. The sub-G_0/G_1 fraction of cells was identified as the apoptotic population.

Annexin V/PI staining

Jurkat cells ($5x10^5$) after Flavin7® exposure were washed twice in PBS and resuspended in 100 μl binding buffer (BDB). The cells were subsequently stained with annexin-V-FITC (AnV) and propidium iodide (PI) (both from BDB) according to the manufacturer's instructions. Immediately after staining, data acquisition was performed as described above. The fata were analyzed using CellQuest Pro software (BDB).

DNA fragmentation Assay

Treated and untreated cells ($1x10^6$) were washed twice in PBS w/o calcium and magnesium. Lysation of cells was performed in a lysis buffer containing 10mmol/L TRIS, 10mmol/L EDTA and 0.5% Triton X-100. Afterwards, proteinase K (1mg/ml) was added and lysate was incubated for 1hour at 37°C. Then, it was heated at 70°C for 10 minutes followed by addition of RNAase (200 μg/ml) and another 1 hour incubation at 37^{o}C. Samples were subsequently transferred to 2% agarose gel and run with 40V at 3 hours. DNA fragments were visualized by UV transluminator by ethidium bromide staining.

Endothelial cells isolation and cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected human umbilical cords by collagenase digestion of the umbilical vein interior according to the methods described by Marin *et al.* (2001). Cells were plated in 100×20 mm tissue culture dishes (Sarstedt, Germany) coated with 1.5% gelatin. Cells were grown to confluence in cultivation medium. Primary cultures were harvested at confluence with 0.05% trypsin-0.02% EDTA (Invitrogen, USA) and plated at a split ratio of 1:3 in tissue culture dishes. The cells were fed with fresh medium one day before each individual experiment. The endothelial identity of the cells was confirmed by their "cobblestone" morphology and CD31 expression. The cells were stained with a combination of CD45-FITC (BDB)/CD31-PE (Caltag, USA) monoclonal antibodies and analyzed by flow cytometry. Endothelial cells were identified as $CD31^{+}CD45^{-}$ cells and represented almost 100 % of all cells in every primary culture.

Endothelial cell migration assay

The migratory activity of HUVECs was assessed using a „wound healing“ assay. Sub-confluent monolayers in 24-well plates were wounded with pipet tips giving acellular 1-mm-wide lane per well. After washing, cells were supplied with 1.5 ml cultivation medium in the absence (controls) or presence of F7. Cells were then allowed to migrate into the wound (empty space produced from the scratch) over a 24 h period. Quantitation of cell migration was performed as described by Li and Cheung (2002).

In vitro angiogenesis assay

The effect of the F7 on the capillary tube formation by endothelial cells was performed using the Fibrin Gel *In Vitro* Angiogenesis Assay Kit (Chemicon, USA) strictly following the manufacturer's instructions. Briefly, HUVECs suspended in the Endomed medium (Biochrom, Germany) supplemented with VEGF and with or withouth F7 were seeded in fibrin-coated 96-well culture plates at concentration of $5x10^3$ HUVECs/100 μl/well. Tube formation was observed periodically over time under a phase contrast microscope. Representative pictures shown in the Results section were taken at 24 h after F7 treatment.

Gelatinase zymography

Matrix metalloproteinases released into conditioned media were determined by gelatinase zymography according to the method of Newcomb *et al.* (2005) with minor modifications. Briefly, cells (5×10^5) were seeded in 6-well plates in 2 ml cultivation medium for 24 h. Then, cells were washed with PBS and incubated in 1 ml of serum-free cultivation medium for additional 24 h in the absence (control) or presence of different dilutions of F7. Medium was collected, clarified of cellular debris by centrifugation for 20 min at 1000 g at –4°C, and stored at –80°C until analyzed. Proteins were electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were renatured in 2.5% Triton X-100 (2 x 15 minutes), then incubated overnight at 37°C in development buffer (50 mM Tris-HCl, pH 7,6; 10 mM $CaCl_2.2H_2O$; 50 mM NaCl; 0,05 % Brij35 [MP Biomedicals]). The gels were stained with 0.5% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for 40 min at room temperature and then distained for 2 h in 50% methanol and 10% acetic acid. Proteolytic activity for MMP-2 in the gel was visualized as clear white bands at 68 kDa against a blue background and for MMP-9 at 92kDa. The gelatinase standard (Chemicon) was used as a positive control for gelatin zymography.

Statistical Analysis

For all experiments, mean values and standard deviations (from 3 experiments) were calculated using the ArcusQuickstat software package. To evaluate the statistical significance observed between groups, Student's *t*-test was employed. The statistical significance was considered to be present if $p<0.05$.

Results

Cytotoxicity assay

Survival of Jurkat as well as HeLa cells exposed to various dilutions of F7 is shown in Tab.1. F7 significantly decreased cell survival at 1/5, 1/10, 1/25 and 1/50 dilutions in both, Jurkat and HeLa cell lines. Reduction in cell viability by ~50% in comparison with the control was achieved at 1/25 dilution of F7 for both cancer cell lines. On the other hand, significant decrease in survival of HUVECS was observed only at 1/5 and 1/10 dilutions (Tab. 1).

Annexin V/PI staining

In the control cells, less than 3.5 % of the early apoptotic cells (AnV^+/PI^-) were detected. Exposure (24 hours) of cells to F7 at 1/25 dilution resulted in an evident increase in the proportion of early apoptotic cells (to 11.7 %). Furthermore, after 48 hours of treatment it was observed a significant increase in the number of early apoptotic cells as well as late apoptotic/necrotic cells (AnV^+/PI^+) and more than 73% of the cells were stained with AnV or PI (Tab.2).

Cell cycle analysis

Jurkat cells exposed to F7 at 1/25 dilution exhibited a time-dependent increase in the sub-G_0/G_1 fraction with the onset already after 24 h. After 48 hours of incubation, more than 60 % of cells exposed to F7 were found as having sub-G_0/G_1 DNA content. These results indicate that F7 causes the appearence of the fraction of cells with sub-G_0/G_1 DNA content which is suggestive of apoptosis (Tab. 3).

DNA fragmentation assay

DNA fragmentation reflecting the endonuclease activity characteristic of apoptosis was also analyzed. As seen in Fig. 1, 72 hours treatment with F7 at 1/5, 1/10, 1/25 and 1/50 dilutions resulted in the formation of DNA fragments that could be visualized via electrophoretic examination as a characteristic ladder pattern.

In vitro angiogenesis assay

To evaluate potential antiangiogenic activity of F7, a bioassay using HUVECs was applied. F7 ability to inhibit capillary tube formation by HUVECs grown on a fibrin gel matrix was examined. HUVECs in the Endomed medium supplemented with VEGF displayed formation of capillary/tube-like networks after 24 hours of incubation at 37°C. F7 completely inhibited capillary tube formation by HUVECs in contrast to vehicle-treated control cultures. It is notable that though the signs of cytotoxicity were observed at dilutions 1/5 and 1/10, much lower concentrations, i.e higher dilutions (1/100 and 1/250) manifested no cytotoxicity while a complete inhibition of tube formation was still observed (Fig .2)

Endothelial cell migration assay

For the determination of potential anti-migratory activities, F7 was tested in a wound closure assay. After wounding with a pipet tip, solvent controls reformed a confluent monolayer within 24 h of incubation. F7 was added in dilutions from 1/10 to 1/500. In the presence of F7, a potent dose-dependent inhibition of endothelial cell migration was observed at dilutions from 1/10 to1/250 (Fig. 3) ($p<0.001$; $p<0.05$).

Gelatinase zymography

To investigate the effect of F7 on MMP-2 and MMP-9 activity, gelatin zymography was performed. Gelatin zymography revealed that F7 reduced MMP-9 activity in HUVECs in a concentration-dependent manner. The active bands of MMP-9 gradually diminished when HUVECs were treated with differentdilutions of F7 (from 1/250 to 1/10). Inhibitory effect of F7 on MMP-2 activity was observed only at the highest concentration (dilution 1/10) (Fig. 4).

Discussion

The use of complementary and alternative medical therapies, particularly in cancer patients, is common (Nam *et al.* 1999). A large variety of herbal products is available in natural health products stores as dietary supplements for cancer patients. However, not much is known about their efficacy, active principles and mode of action.

Flavin7® is derived from concentrated extracts of seven fruits (grape, blackberry, black cherry, black currant, elderberry, blackthorn and plum) chosen for their high concentration of bioflavonoids (e.g. quercetin, kaempferol, chrysin, rutin, catechin, galangin, apigenin, luteolin) and resveratrol. Although individual components of F7 have antioxidant, antiestrogenic, antitumor and many other properties, *in vitro* evidence of anticancer activity for this polyphenol complex itself is still lacking.

Our *in vitro* studies demonstrated that F7 significantly suppresses viability of cancer cells. To examine the mechanism that might account for the effects of F7 in cancer cells, we investigated its effects on cell cycle distribution. We observed F7-induced reduction in the proportion of cells in the S and G_2/M phases with concomitant increase in the fraction of cells with sub-G_0/G_1 DNA content which is considered to be a marker of apoptotic cell death. Apoptosis was also confirmed by annexin V/PI staining. In addition, DNA fragmentation was also clearly present as determined by agarose gel electrophoresis.

As it was mentioned above, the precise mechanism of F7 antiproliferative effect has not been elucidated yet. On the other hand, many researchers have conducted *in vitro* as well as *in vivo* studies on the potential anticancer activity of flavonoids and resveratrol in diverse experimental systems (Kuntz *et al.* 1999, Caltagirone *et al*. 2000, Yanez *et al.* 2004, Fulda and Debatin, 2006).

Flavonoids have been shown to induce apoptosis in cancer cell lines (Zhao *et al.* 2006), to inhibit activities related to tumor promotion, proliferation and mitotic signal transduction (Lee and Lin 1997) or to cause cell cycle arrest (Hastak *et al.* 2005).

Resveratrol (3,5,4´-trihydroxystilbene) is a naturally occurring phytoalexin produced by a wide variety of plants such as grapes, peanuts, mulberries in response to stress, injury, UV irradiation, and fungal infection. Although phytoalexins have long been inferred to be important in the defense of plants against fungal infection, numerous reports indicate that resveratrol has also anticancer effects (Aggarwal *et al.* 2004, Barta *et al.* 2006).

Angiogenesis is a highly regulated process that involves a complex cascade of events and play an important role in tumor growth and metastasis. It is a key process in the promotion of cancer (Folkman, 2003). It was documented that many natural health products inhibit angiogenesis. Among the known „natural“ angiogenesis inhibitors, polyphenols seem to play an important role (Foits *et al.* 1997, Kanadaswami *et al.* 2005). However, the mechanism behind the antiangiogenic effect of polyphenols is unclear. In our experiments we found that F7 significantly suppressed some of the basic steps in angiogenesis such as growth, migration and capillary tube formation by HUVECs as well as MMPs gelatinolytic activity.

Matrix metalloproteinases, particularly MMP-2 and -9, play a crucial role in tumor invasion and angiogenesis. They are enzymes that break down extracellular matrix proteins to allow further differentiation and spread of endothelial cells during angiogenesis. Several reports indicate that MMPs play a major regulatory role in the initiation of angiogenesis (Folkman, 2006). Recent evidence from another laboratory has indicated the involvement of MMPs in the early stages of morphogenesis of endothelial cells on fibrin matrix (Collen *et al.*, 2003). In this report, we demonstrate that F7 differentially regulated the activity of MMP-2 and

MMP-9. Our results are consistent with other studies showing ability of flavonoids as well as resveratrol to inhibit different MMPs (Vijayababu *et al*., 2006). These effects of the above mentioned polyphenols may partly explain our observation of inhibition of endothelial cells migration and capillary tube formation.

In conclusion, the present report describes for the first time potential anticancer effects of F7. We observed a marked *in vitro* effect of F7 on the growth, cell cycle and apoptosis of human tumor cell lines. Additional mechanisms such as effects on growth, migration, tube formation by endothelial cells as well as MMPs inhibition were also observed. The detected pronounced *in vitro* activity of F7 suggests that it may possess its own potent anticancer effects. Additionally, our results also suggest that some of the F7 constituents, either independently or in combination, may affect the process of neovascularisation.

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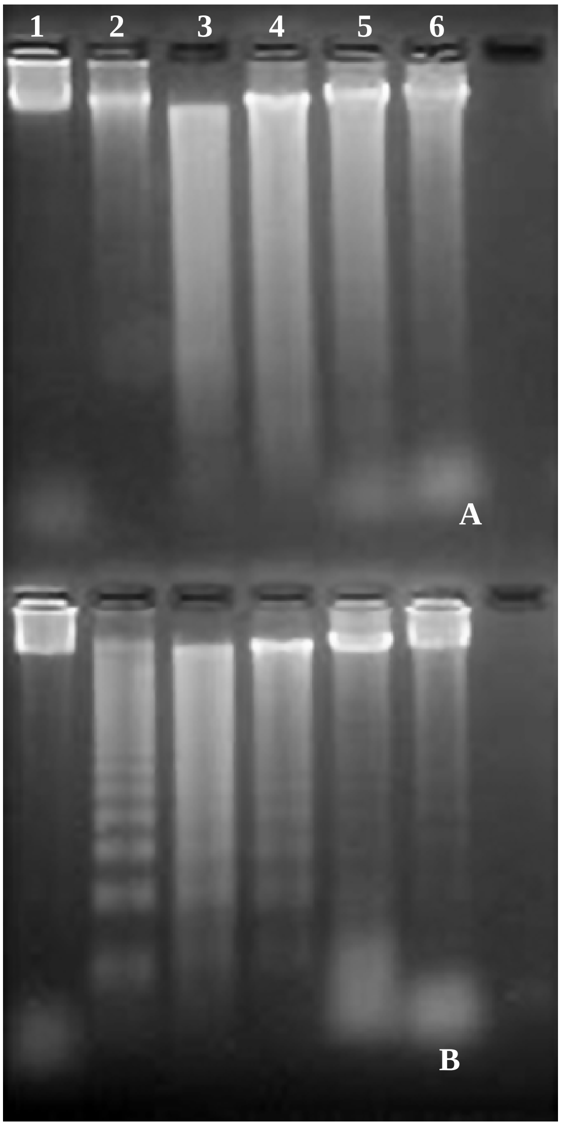
Fig. 1. The effect of F7 on the presence of DNA fragments in Jurkat (A) and HeLa (B) cells incubated for 72 hours. DNA fragmentation was analyzed by electrophoresis in a 2.0 % agarose gel. Lanes indicate different treatments: control (lane 1), positive control (etoposide 50 $\mu l.ml^{-1}$ for 72 hours) (lane 2), F7 at 1/5 dilution (lane 3), F7 at 1/10 dilution (lane 4), F7 at 1/25 dilution (lane 5) and F7 at 1/50 dilution (lane 6)

Fig.2. Inhibition of capillary-like tube formation in human umbilical vascular endothelial cells by F7 in fibrin-coated culture plates. Human umbilical vascular endothelial cells were suspended in Endomed medium supplemented with VEGF and were plated onto a layer of fibrin at a density of 51×10^3 cells/well without (A) or with F7 at 1/50 (B), 1/100 (C) or 1/250 dilution (D). The method is described in Materials and methods. (magnification: ×100).

Fig. 3. Effect of F7 on HUVECs migration. Percentage of remaining scratched area was calculated after being marked and quantified by the histogram function of the Adobe Photoshop 5.5 program. Experiments were performed in triplicate. *** $p<0.001$, *$p<0.05$

Fig 4. Inhibition of MMP-2 and MMP-9 activity. Conditioned medium of HUVEC treated with F-7 (1/10-1/250 dilution) was harvested after 24 h and subjected to non-reducing SDS–PAGE through a 10% acrylamide resolving gel containing 1 mg/ml gelatin as described in Methods.

Flavin7 decreased gelatinolytic activity of enzyme MMP-9 as it is indicated by clear bands in the gel at lanes 2–6: F-7 (1/10, 1/25, 1/50, 1/100 and 1/250). Line 1 is control and lane 6 is MMP-9 and MMP-2 standard. This is a representative gel picture of one of three separate experiments with similar results.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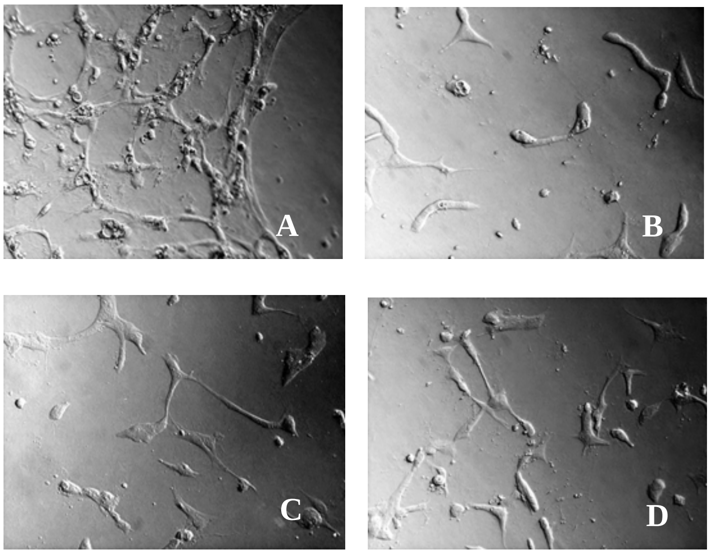
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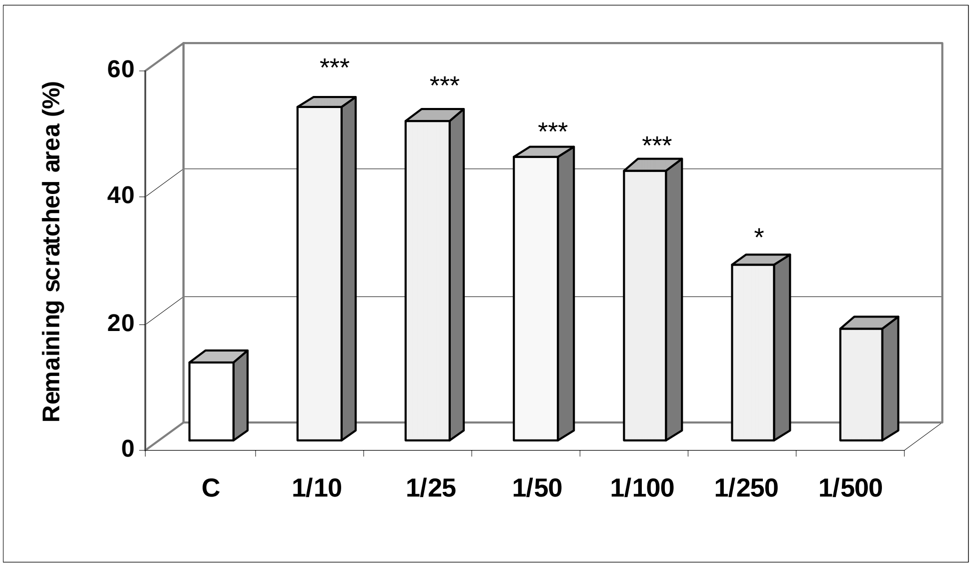
Remaining scratched area (%)
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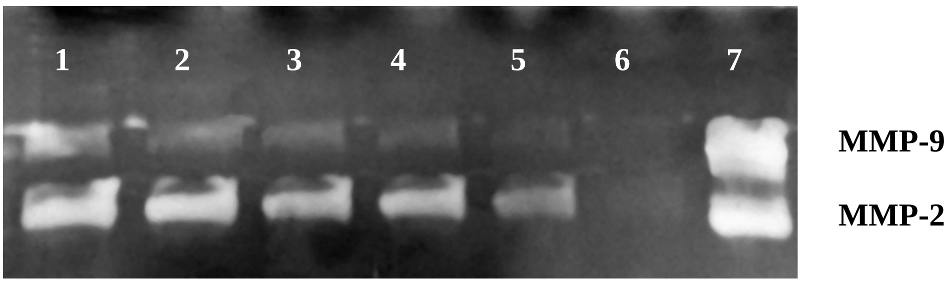
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MMP-9
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Tab.1. ***In vitro*** **cytotoxicity data of F7 by the MTT assay (in %).**

*** $p<0.001$; ** $p<0.01$ vs untreated cells (control)

Tab. 2. F7-induced apoptosis in Jurkat cells measured by flow cytometry.

Time (hr)	An-/PI-	An+/PI-	An+/PI+
Control	96.7	0.4	2.1
24	36.5	9.7	44.9
48	19.3	11.5	63.6

AnV^-/PI^- - live cells; AnV^+/PI^- - early apoptic cells; AnV^+/PI^+ - late apoptotic/necrotic cells
Cells were exposed to F7 at dilution 1/25 for 24 and 48 hours

Tab. 3. Flow cytometric analysis of cell cycle distribution in Jurkat cells treated with F7 at 1/25 dilution (in %).

Time (hr)	sub-G_0/G_1	G_0/G_1	S	G_2/M
Control	1.6	50.3	30.4	17.4
24	37.1	33.7	19.9	9.2
48	60.3	24.7	13.0	2.1