

## Comparative Anticancer Effects of Flavonoids and Diazepam in Cultured Cancer Cells

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**This study examined the comparative anticancer effects of flavonoids and diazepam in the cultured cancer cells. In the SNU-C4 colorectal and MDA-MB-231 breast adenocarcinoma cells, apigenin and fisetin, flavonoids, and diazepam inhibited cancer cell survival concentration and incubation-time dependently. Diazepam consistently inhibited FAS activity, a known anticancer mechanism of flavonoids, in a concentration dependent manner. Unlike diazepam, in highly aggressive breast MDA-MB-231 cells known to have a nuclear/perinuclear located PBR, PK11195, a specific PBR ligand enhanced the proliferation of cells, and the proliferative effect of PK11195 was reversed by an addition of lovastatin, a HMG-CoA reductase inhibitor. Diazepam- and flavonoids-induced cytotoxic activity in both cancer cell lines was not reduced by the addition of 5-fluorouracil (5-FU), a chemotherapeutic agent. Like flavonoids, diazepam inhibited the release of vascular endothelial growth factor (VEGF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) into supernatants of cultured in the SNU-C4 and MDA-MB-231 cells. In conclusion, this study provided *in vitro* information on the safe use of sedative in oncologic patients.**

**Key words** flavonoid; diazepam; *in vitro* anticancer effect; fatty acid synthase; vascular endothelial growth factor; granulocyte-macrophage-colony stimulating factor

The safe use of sedative is often important for oncologic patients. Anticancer chemotherapeutic agents have been reported to reduce cognitive function *via* hippocampal neuronal toxicity, and increase the vulnerability for development of depression during certain stressful periods.<sup>1–3</sup> Especially in the case of geriatric patients receiving a palliative care, cautions should be paid. In brain aging, brain area involved in the modulation of the hypothalamo-pituitary-adrenal (HPA) axis might be significantly changed. Late-life depression and anxiety may lead individuals to be unable to adjust their physiology and behavior to stressful events and elevate their circulating cortisol to chronic levels, with detrimental consequences to adversely altered HPA axis.<sup>4,5</sup> Efforts toward stabilization of altered HPA axis *via* antianxiety and antidepressant effects, even in mild or transient cases, may affect favorably on the progress of aged patients having malignancy. It has also been suggested that the drug can play anticancer function by inhibiting proliferation of some cancer cell lines.<sup>6–8</sup> Thus, for the control of anxiety and depression, it is reasonable to suppose that use of sedative may be one of choices for the management of malignancies, especially in geriatric patients. However, prolonged use of synthetic benzodiazepines like diazepam, is limited owing to harmful adverse effects including tolerance.

Diazepam have been known to bind the peripheral-type benzodiazepine receptors (PBRs), and PBR ligands, including PK11195, have been reported to enhance apoptosis and elicit cell cycle arrest in many types of tumors.<sup>9–14</sup> However, cancer progression has also been implied in cancer cells with perinuclear/nuclear located PBRs, especially certain types of breast cancer cell, and their proliferative effect linked to a cholesterol influx into the nuclear membrane.<sup>15,16</sup>

Accordingly, to establish scientific information for the safe use of sedative in the case of malignancies, this study examined the *in vitro* anticancer characteristics of diazepam and

flavonoids.

### MATERIALS AND METHODS

**Materials** The human adenocarcinoma SNU-C4 (colorectal) cancer cell line was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea), while the human breast adenocarcinoma MDA-MB-231 cell lines were kindly provided by Dr. Sang-Hyun Kim (Kyungpook National University, School of Medicine). The RPMI medium 1640, trypsin solution, sodium pyruvate, ethylene glycol-bis-(aminoethylether) *N,N,N',N'*-tetraacetic acid, feta bovine serum (FBS) and antibiotics (penicillin and streptomycin) were all purchased from GIBCO (Grand Island, NY, U.S.A.), the diazepam kindly donated by Roche (Switzerland), the petroleum benzin purchased from Fluka (Germany), and the Lovastatin purchased from Tocris Cookson Inc. (MI, U.S.A.). All the other assay reagents and chemicals, including apigenin were purchased from Sigma (St. Louis, MO, U.S.A.). The diazepam, PK11195 (1-[2-chlorophenyl]-*N*-methyl-*N*-[1-methylpropyl]-3-iso-quinolinecarboxamide), apigenin, and fisetin were prepared as stock solutions in 100% ethanol or methanol, then diluted with an aqueous medium to the final desired concentrations. The stock solutions of drugs were sterilized by filtration through 0.22  $\mu$ m disc filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) before being applied to the cells.

**Cell Culture** The cells were cultured at 37 °C in a humidified incubator under 5% CO<sub>2</sub>/95% air in an RPMI 1640 medium supplemented with 10% FBS, 200 IU/ml penicillin, 200  $\mu$ g/ml streptomycin and 1 mM sodium pyruvate. The culture medium was replaced every 2–3 d.

**MTS Assay** The cytotoxicity was measured using an MTS assay. The cells ( $5 \times 10^4$ ) were seeded in wells containing 100  $\mu$ l of the RPMI medium supplemented with 19%

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FBS, in 96-well plates. After 24 h, various concentrations of different materials were added. After 24, 72 and 144 h, 20  $\mu$ l of an MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) solution (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay, Promega) was added and the plates were incubated for further 1–4 h. The optical density at 490 nm was measured with a 96-well plate reader, as the quantity of formazan produced, as measured by the absorbance at 490 nm, is directly proportional to the number of living cells in the culture. Concentration for 50% inhibition ( $IC_{50}$ ) values were obtained *via* nonlinear regression of concentration–response curves.

**Fatty Acid Synthase (FAS) *in Vitro* Assay** The cells were harvested and the pellets collected by centrifugation. The resulting pellets were then resuspended in a hypotonic buffer (pH=7.5) consisting of 1 mM EDTA and 20 mM Tris–HCl. The sample protein contents were measured using a BCA assay kit (Pierce). 50  $\mu$ g protein, various concentrations of drugs and 20  $\mu$ l of the reaction mixture were then added to tubes containing 2.5 ml of a potassium phosphate buffer (100 mM, pH=7). The reaction mixture consisted of 2.5 mM NADPH, 1.25 mM acetyl-CoA, 1.25 mM Malonyl-CoA and 0.02 mM [2-<sup>14</sup>C]malonyl-CoA (45 mCi/mmol, BMS). The tubes were incubated for 15 min, at 37 °C. The reaction was stopped by adding 3 ml of ice-cold 1 M HCl/methanol (6 : 4 v/v). The fatty acids were extracted with petroleum benzin. The incorporation of [2-<sup>14</sup>C]malonyl-CoA activity was measured with a scintillation counter (Wallac, Finland).

**Measurement of Vascular Endothelial Growth Factor (VEGF) and Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF)** After trypsinization process,  $3 \times 10^6$  cells were placed in the assay wells of culture dishes containing 1 ml of complete RPMI (Falcon multiwell 12 wells, Becton Dickinson Labware, U.S.A.). Then, cells were treated with  $10^{-6}$  M concentration of apigenin, fisetin and diazepam for 6 d, respectively. Human vascular endothelial growth factor kit (VEGF Immunoassay, Koma-biotech, Korea) and human granulocyte-macrophage-colony stimulating factor kit (GM-CSF Immunoassay, Komabiotech, Korea) were used to measure VEGF and GM-CSF concentration, respectively. The optical density of each sample was measured in microplate reader at 450 nm. Release amounts of VEGF and GM-CSF were normalized by viable cell counts in each well.

**Statistical Analysis** The data represent the mean  $\pm$  the standard error (S.E.). Inter-group data comparisons were made using Student's *t*-test (Systat, Intelligent Software, Evanston, IL, U.S.A.). Nonlinear regression for obtaining the  $IC_{50}$  value were made using computer software (Prism, Graphpad, U.S.A.).

## RESULTS

As shown in Fig. 1, treatment for 72 h with apigenin and fisetin, both flavonoids showed concentration-dependent inhibition on the survival of the SNU-C4 and MDA-MB-231 adenocarcinoma cells. In the SNU-C4 cells,  $IC_{50}$  ( $\mu$ M) of apigenin- and fisetin-treated group was  $2.3 \pm 0.2$  and  $0.8 \pm 0.1$ , respectively. In the MDA-MB-231 cells,  $IC_{50}$  ( $\mu$ M) of apigenin- and fisetin-treated group was  $1.2 \pm 0.3$  and  $3.2 \pm 0.5$ ,

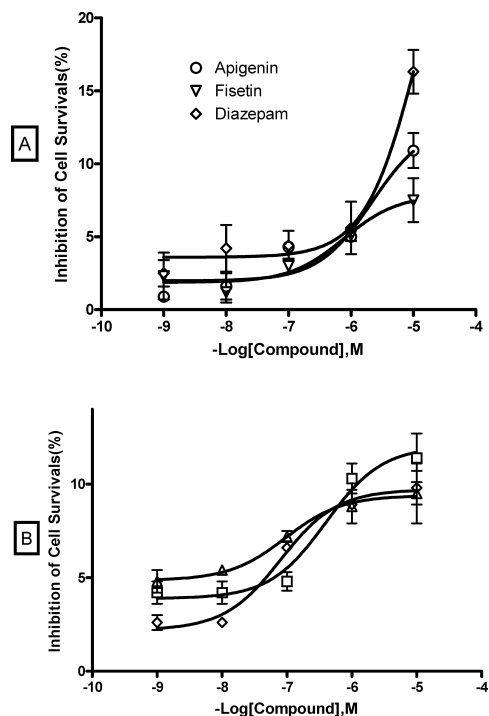


Fig. 1. Concentration–Responses of Apigenin, Fisetin and Diazepam on Cancer Cell Survivals

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Data represent mean  $\pm$  S.E. of eight experiments. Various concentrations ( $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M,  $10^{-10}$  M) of drugs were treated for 72 h, respectively.  $IC_{50}$  values were obtained *via* nonlinear regression of concentration–response curves.

respectively. Same treatment of diazepam, a synthetic benzodiazepine, showed also cancer cell survival concentration-dependent.  $IC_{50}$  ( $\mu$ M) of diazepam-treated group was  $5.5 \pm 1.2$  and  $0.12 \pm 0.1$ , in the SNU-C4 and MDA-MB-231 adenocarcinoma cells, respectively.

As shown in Fig. 2, in the longer duration of treatment, micromolar concentration of apigenin and fisetin showed the higher inhibitory effect on the survival of the SNU-C4 and MDA-MB-231 adenocarcinoma cells. Like flavonoids,  $10^{-6}$  M concentration of diazepam, also showed incubation time-dependent inhibition of the cell survival. In the group treated for 72 h with the same concentration of apigenin, fisetin and diazepam, inhibition of cell survival (%) were increased to  $10.3 \pm 1.0$ ,  $8.8 \pm 0.9$  and  $8.9 \pm 1.2$ , respectively. In the group treated for 144 h with the same concentration of apigenin, fisetin and diazepam, inhibition of cell survival (%) were increased to  $9.2 \pm 1.2$ ,  $10.9 \pm 2.1$  and  $9.6 \pm 0.6$ , respectively.

As shown in Fig. 3, treatment for 24 h with a  $10^{-6}$  M concentration of PK11195, a PBR ligand, slightly inhibited the survival of the SNU-C4 colorectal cancer cells, while for the MDA-MB-231 breast cancer cells, the same concentration of PK11195 enhanced the cell survival to  $20.1 \pm 2.1\%$ . However, the addition of a  $10^{-6}$  M concentration of lovastatin, a HMG CoA reductase inhibitor, reversed the proliferative effects of PK11195 in the MDA-MB-231 cells, and lovastatin increased the cytotoxic effects of PK11195 in the SNU-C4 cells. Single treatment of  $10^{-6}$  M concentration of lovastatin for 24 h showed weak inhibitory effect on the cancer cell survival, and inhibition of cell survival (%) were  $4.5 \pm 0.1$  and

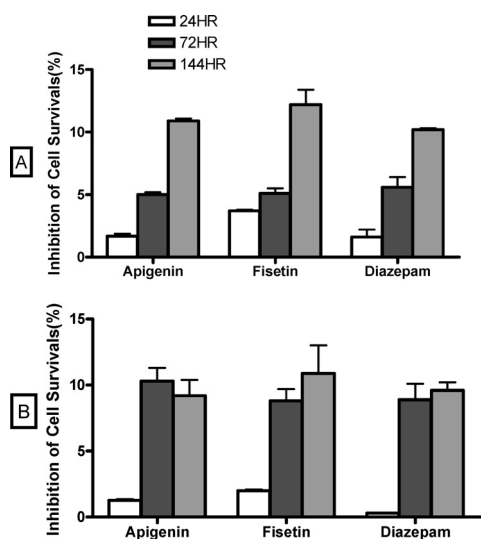


Fig. 2. Modulatory Effects on the Cancer Cell Survivals by Apigenin, Fisetin and Diazepam

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Data represent mean  $\pm$  S.E. of eight experiments.  $10^{-6}$  M concentration of drugs was treated for 24, 72 and 144 h, respectively.

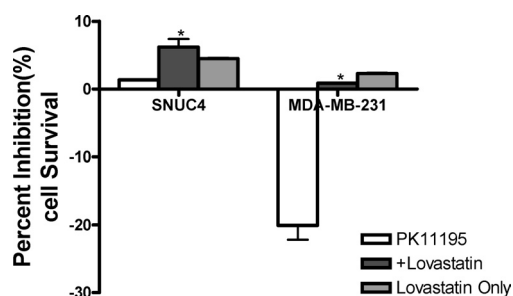


Fig. 3. Modulatory Effects on the Cancer Cell Survivals by PK11195 and/or Lovastatin, a HMG-CoA Reductase Inhibitors

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Data represent mean  $\pm$  S.E. of eight experiments. \*  $p < 0.05$ : significantly different from PK11195-treated groups.  $10^{-6}$  M concentration of drugs was treated for 24 h.

$2.3 \pm 0.1$  in the SNU-C4 and MDA-MB-231 cancer cells, respectively.

As shown in Fig. 4, concentration-dependent inhibitory effect on FAS activity, known as an anticancer mechanism of flavonoids, was also observed in the various concentrations ( $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M,  $10^{-8}$  M) of the sedative-treated (for 30 min) SNU-C4 and MDA-MB-231 cancer cells. In the apigenin, fisetin and diazepam-treated groups,  $IC_{50}$  ( $\mu$ M) were  $3.9 \pm 0.6$ ,  $1.0 \pm 0.2$ , and  $5.4 \pm 0.4$ , respectively for the SNU-C4 cells, and  $0.6 \pm 0.1$ ,  $4.7 \pm 0.6$ , and  $2.4 \pm 0.3$ , respectively for the MDA-MB-231 cancer cells.

As shown in Fig. 5, for the SNU-C4 and MDA-MB-231 adenocarcinoma cells, anticancer cytotoxicity induced by flavonoids or diazepam was not reduced by treatment of  $10^{-6}$  M concentration of 5-fluorouracil (5-FU), a chemotherapeutic agent. Their inhibitory action on cancer cell survivals was further enhanced by the addition of PK11195, a putative chemosensitizer.

As shown in Fig. 6, treatment of micromolar concentration of apigenin and fisetin reduced the release of VEGF, and examined flavonoids also reduced the release of GM-CSF from

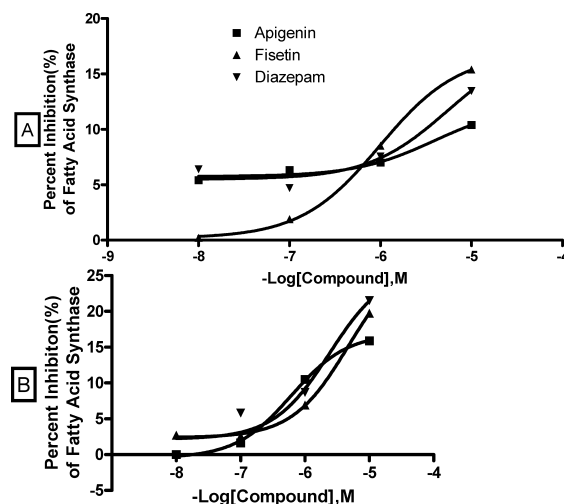


Fig. 4. Inhibitory Effects on the Fatty Acid Synthase Activity by Apigenin, Fisetin and Diazepam

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Data represent mean  $\pm$  S.E. of eight experiments. Various concentrations ( $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M,  $10^{-8}$  M) of drugs were treated for 30 min, respectively.  $IC_{50}$  values were obtained via nonlinear regression of concentration-response curves.

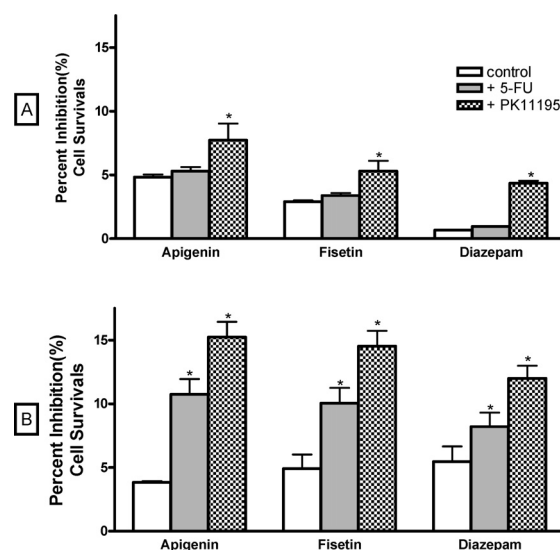


Fig. 5. Enhancement of Apigenin, Fisetin and Diazepam on the 5-Fluorouracil (5-FU)-Induced Cytotoxicity

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Data represent mean  $\pm$  S.E. of eight experiments. \*  $p < 0.05$ : significantly different from control.  $10^{-6}$  M concentration of drugs was treated for 24 h.

the SNU-C4 and MDA-MB-231 adenocarcinoma cells. In this study, like flavonoids, micromolar concentration of diazepam inhibited the release of VEGF and GM-CSF into supernatants of cultured SNU-C4 and MDA-MB-231 adenocarcinoma cells.

## DISCUSSION

Flavonoids, as polyphenolic compounds found in plants, have already been reported to have anti-cancer efficacies.<sup>17–19</sup> Consistently, in this study, apigenin and fisetin, flavonoids showed *in vitro* anticancer cytotoxicity. Like flavonoids, diazepam, a sedative also showed inhibition of cancer cell survival. The anticancer cytotoxicity of flavonoids and diazepam

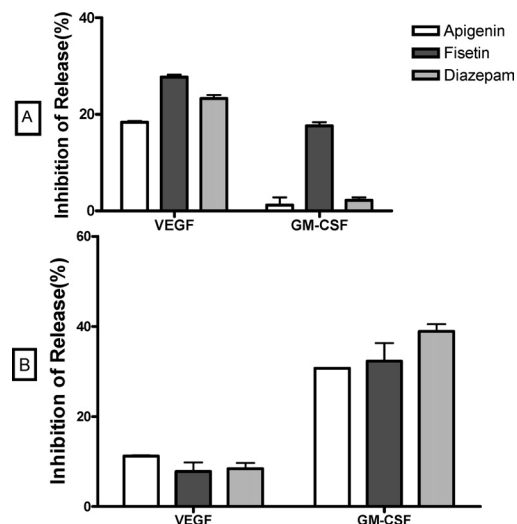


Fig. 6. Effects of Apigenin, Fisetin and Diazepam on the Release of Vascular Endothelial Growth Factor (VEGF) and Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) in Cancer Cells

A: SNU-C4 human colon adenocarcinoma cells, B: MDA-MB-231 human breast adenocarcinoma cells. Drugs and vehicle (for the control) were treated for 6 d. Data represent the mean  $\pm$  S.E. of 6 experiments.

showed the concentration-dependency, and micromolar ranges of  $IC_{50}$ . The plasma concentration of diazepam in patients who are taking therapeutic dose of diazepam (5–10 mg) were found to be micromolar range. The inhibition of FAS activity, is a known cytotoxic mechanism of flavonoids,<sup>20)</sup> and in the present study, micromolar concentration of diazepam consistently inhibited the FAS activity in the SNU-C4 and MDA-MB-231 cells.

In highly aggressive breast cancer cells, such as MDA-MB-231 cells, a nuclear/perinuclear located PBR have been reported to mediate the stimulation of cancer cell survival.<sup>15,16)</sup> Consistently, in this study, PK11195, a specific PBR ligand remarkably enhanced the proliferation of MDA-MB-231 breast adenocarcinoma cells, but diazepam reduced the cell survival. However, PBR as a critical part of the mitochondrial permeability transition pore (mPT) has been shown to mediate enhancing the apoptosis.<sup>9–14)</sup> In a previous study by the current authors,<sup>21)</sup> anticancer cytotoxic flavonoids including apigenin elicited up-regulation of PBR mRNA expression in SK-N-Mc human neuroblastoma cells. Moreover, in this study, treatment with PBR ligands consistently inhibited the SNU-C4 cell survival, and diazepam also produced anticancer cytotoxic effects, similar to those caused by flavonoids.

In highly aggressive cancer cells, including MDA-MB-231 breast cancer cells, an increased cholesterol influx into the nucleus has been reported to be associated with the proliferative action of a perinuclear/nuclear located PBR.<sup>16)</sup> Consistent with this, in the present study, proliferative effect of PK11195 on of the MDA-MB-231 cells was reversed by the co-administration of lovastatin, a HMG-CoA reductase inhibitor. HMG-CoA reductase is an enzyme that catalyzes the rate-limiting step of the isoprenoid producing mevalonate pathway, and isoprenoid is involved in the activation of Ras and cholesterol synthesis. In particular, lipid soluble statins have been also shown to produce antitumor effects, and reduce intracellular cholesterol level.<sup>22–24)</sup> In the present study,

single treatment of lovastatin showed weak anticancer cell cytotoxicity.

Moreover, the micromolar concentration of diazepam- and flavonoids-induced cytotoxic activity in the cancer cells in this study was not reduced by the addition of 5-fluorouracil (5-FU), a chemotherapeutic agent. PK11195 has been reported to generate a reversal action on the chemoresistance in cancer cells, independent of the direct activation of PBR.<sup>25–27)</sup> In this study, PK11195, a putative chemosensitizer, showed further enhancement of their anticancer effects.

VEGF and GM-CSF were shown to stimulate malignant tumor cell growth and migration *in vitro* and to promote cancer progression *in vivo*.<sup>28,29)</sup> Modulation of angiogenesis *via* VEGF system by black tea polyphenols has been proposed as a mechanism of chemoprevention of rat mammary carcinogenesis.<sup>29)</sup> Flavonoids, active ingredients isolated from several plants, have been also introduced to inhibit tumor angiogenesis through decreasing VEGF expression and the release of the angiogenic peptide VEGF from U-343 and U-118 human glioma cells.<sup>30,31)</sup> Consistently with these, treatment of micromolar concentration of apigenin and fisetin reduced the release of VEGF, and examined flavonoids also reduced the release of GM-CSF from in the SNU-C4 and MDA-MB-231 cells. In this study, micromolar concentration of diazepam inhibit the release of VEGF and GM-CSF like flavonoids into supernatants of cultured in the SNU-C4 and MDA-MB-231 cells.

In cancer cells, increased glucose transporter (Glut) and hexokinase (HK) activities facilitate glucose utilization and thus continuous intracellular accumulation of fluoro-deoxyglucose (FDG). The logic behind delayed FDG PET imaging is based on the known fact that most types of malignant cells have significantly increased ratios of HK to glucose-6-phosphatase activities, which allow 18F-deoxyglucose-6-phosphatate to accumulate to a much higher level over time than in the normal cells. It has been reported that the routine use of 5 mg diazepam *p.o.* before intravenous administration of FDG renders PET imaging ineffective.<sup>32)</sup> It has also been reported that diazepam inhibit the function of Glut, insulin sensitivity and HK activity.<sup>33,34)</sup> From above reports, it can be postulated that sedative may elicit *in vivo* anticancer efficacy.

Agents that inhibit glucose uptake should decrease the proliferation of cancer cells. Certain flavonoids inhibit glucose uptake in culture cells.<sup>35,36)</sup> Flavonoids, polyphenolic compounds distributed widely in plant-based foods, exert diverse biological effects in cultured cells and *in vivo*. Certain flavonoids including apigenin have been reported to show a selective anxiolysis in mice (10 mg/kg *i.p.*), and a partial agonistic activity at the CBRs in micromolar concentration.<sup>37)</sup> In the plasma samples of population taking apigenin-rich foods regularly, similar amounts of the compound were detected. Yet, the difficulties involved with attaining a sufficient concentration to activate the central nervous system present an obstacle. Therefore, the improved activities shown by certain synthetic derivatives, such as methylapigenin, may provide a better challenge. Moreover, caffeic acid butyl ester and various related synthetic nitroflavone derivatives have exhibited potent anticancer activity, without affecting normal cell survival.<sup>38)</sup> Consistently, these options may support a safer use of sedative in the management of oncologic patients. Plus compounds derived from natural products have

been shown to be advantageous over synthetic sedatives, due to diminished adverse effects, such as muscle relaxation, tolerance, and dependency.

The results of our study can increase the knowledge about the influence of the examined substances on proliferation and progression of cancer cells. Our observations might give an insight into the safe use of sedatives in the malignancies. However, for a prolonged use, natural substance like apigenin might be advantageous over a synthetic drugs. In conclusion, this study provided *in vitro* information on the use of flavonoids and sedative in oncologic patients.

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