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Regular paper

The effect of luteolin 7-glucoside, apigenin 7-glucoside and *Succisa pratensis* extracts on NF-κB activation and α-amylase activity in HepG2 cells*

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The chemical composition of *Succisa pratensis* is not well known. The existing data indicate a substantial content of flavonoids, which include luteolin and apigenin 7-glucosides. The aim of this study was to elaborate the isolation protocol of these flavonoids from flowers and leaves of *S. pratensis*, to carry out their characterization, as well as evaluate the effect of *S. pratensis* extracts on activation of transcription factor NF-κB and α-amylase activity. The extraction protocol applied in this study allowed isolation and characterization of flavonoid fraction of *S. pratensis*. Their identity was confirmed by NMR spectra analysis, UV spectroscopy and electrospray ionization-tandem MS evaluation. Treatment of pancreatic α-amylase with *S. pratensis* extract inhibited this enzyme's activity to an extent comparable to that of isolated luteolin and apigenin 7-glucosides. Incubation of HepG2 cells for 24 h with *S. pratensis* extracts or isolated flavonoids resulted in moderate reduction in NF-κB transcription factor activation evaluated in terms of translocation of its active subunits from cytosol into nucleus and subsequently diminished expression of the *COX-2* gene. Expression of NF-κB was also reduced. The most significantly diminished NF-κB activation and expression, as well as *COX-2* expression, was found to result from treatment with isolated flavonoids and ethyl acetate extract of *S. pratensis* leaves. These results indicate that *S. pratensis* flavonoids may modulate the metabolic and signaling pathways whose deregulation is related to pathogenesis of liver cancer. Further studies are required to confirm these observations and assess the chemopreventive and/or therapeutic potential of the *S. pratensis* herb.

Key words: *Succisa pratensis*, apigenin 7-glucoside, luteolin 7-glucoside, α-amylase inhibitory activity, NF-κB

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Abbreviations: (1), luteolin 7-glucoside; (2), apigenin 7-glucoside; COX-2, cyclooxygenase-2; NF-κB, nuclear factor-kappa B; HCC, hepatocellular carcinoma; HepG2 cells, human hepatocellular carcinoma cells; SK1, methanolic extract of *S. pratensis* flowers; SK2, ethyl acetate extract of *S. pratensis* flowers; SL1, methanolic extract of *S. pratensis* leaves; SL2, ethyl acetate extract of *S. pratensis* leaves

INTRODUCTION

Flavonoids are commonly found in plants and they are widely used in medicine, either as isolated compounds or in the form of extracts from plants in which they are abundantly present. Several studies had shown that flavonoids, such as apigenin and luteolin, have antioxidant, anticancer, and anti-inflammatory activities (Choi *et al.*, 2007; Shukla & Gupta, 2010; Bouzaiene *et al.*, 2016; Xue *et al.,* 2017; Yan *et al.,* 2017). Moreover, *in vitro* studies demonstrated that anti-inflammatory properties of luteolin may be mediated by inhibition of the nuclear factor-κB (NF-κB). This transcription factor is a regulator of cell survival, immunity and inflammation. NF-κB p50-p65 subunits represent the major active complex in most of the cells. Normally, NF-κB is sequestered in the cytosol by its inhibitor, the IκB protein. NF-κB stimulation leads to activation of the IκB kinase (IKK) and subsequently activation of gene transcription, such as *COX-2*, encoding *cyclooxygenase-2* (Hwang *et al.*, 2011; Chung *et al.,* 2012). Numerous reports indicated that the observed effect of luteolin on COX-2 and inducible nitric oxide synthase (iNOS) suppression is due to inhibition of NF-κB (Hu & Kitts, 2004; Chung *et al.*, 2012; Kiraly *et al.*, 2016). Moreover, luteolin 7-glucoside and apigenin 7-glucoside also inhibit the α-amylase activity. This effect depends on the amount and substitution position of hydroxyl groups in the molecule and the presence of sugar substituents (Han *et al.*, 2003; Tadera *et al.,* 2006; Asgharia *et al.*, 2015; Zhang *et al.*, 2017).

Inhibition of α-amylase limits the availability of glucose, which might affect the cancer cell survival (Palorini *et al.,* 2016).

In our earlier report we showed that in *Succisa pratensis* herb's methanolic extract, there is a substantial amount of flavonoids, including luteolin and apigenin 7-glucosides (Witkowska-Banaszczak & Długaszewska, 2017).

The aim of this study was isolation and identification of flavonoids from *S. pratensis* flowers and leaves, and comparison of the effect of isolated luteolin and apigenin 7-glucosides with different plant extracts containing the whole flavonoids' fraction on the α-amylase activity in a cell-free system. Moreover, the influence of these preparations on activation and expression of NF-κB in human hepatocellular carcinoma cells (HepG2 cells) was evaluated.

MATERIALS AND METHODS

Plant material. *S. pratensis* was cultivated in the field of the Department of Medical and Cosmetic Natural

Products, Poznan University of Medical Sciences, Mazowiecka 33, 60-623 Poznan. The leaves were collected in July 2016 and dried. The voucher number of the plant was 1104.

Preparation of extracts. The dried (10 g) leaves or flowers of *S. pratensis* were extracted with 200 mL of methanol and ethyl acetate for 30 min using an ultrasonic bath. The extraction was carried out at 50°C, 1000 W ultrasonic power, frequency of 37 kHz (Elma S180 H). The methanolic and ethyl acetate extracts were concentrated to dry mass and used to prepare concentration suitable for the α-amylase inhibitory test and HepG2 cells' exposure.

Isolation and identification of flavonoids. The flavonoids were isolated from a methanol-water (1:1) *S. pratensis* flower extract using column chromatography.

The column was filled with cellulose Wathman CF11 (Whatman, Germany) and eluted with ethyl acetate-methanol-water (100-5-5) as a solvent system, and next, the eluate was transferred on the Sephadex LH-20 column (25–100 μm, Sigma-Aldrich, Germany) and subsequently eluted with methanol.

Thin-layer chromatography (TLC) was used to control the isolation process [cellulose plates (Merck, Germany) with CH_3COOH-H_2O (15:85); silica gel 60 plates (Merck, Germany) with EtOAc-HCOOH-H_2O (100:11:11:26)]. The flavonoid compounds were observed under UV at 366 nm before and after visualization by 1 % Naturstoffreagenz A in MeOH (NA).

The identification of the compounds was performed on the basis of UV, ESI-MS,^{1}H and ^{13}C NMR analyses. The NMR spectra (^{1}H, ^{13}C NMR) were recorded using a Bruker NMR Avance II 400 MHz spectrometer, CD_3OD with TMS as an internal standard. The ESI – MS mass spectra were measured on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer. The obtained data were comparable to the published values (Giang & Son, 2004; Gohari *et al*., 2011; Peng *et al.,* 2016). The UV spectra were recorded on a UV/VIS Perkin Elmer Lambda 35 spectrophotometer in MeOH, and also after addition of specific reagents ($NaOAc/H_3BO_3$, $AlCl_3$, $AlCl_3$/HCl, NaOMe, NaOAc), according to Mabry and co-workers (Mabry *et al*., 1970).

For the biological assay, the methanolic or ethyl acetate extracts from the flowers (SK1, SK2) and leaves (SL1, SL2) were applied.

Pancreatic α-amylase (EC.3.2.1.1) **activity inhibition assay**. The α-amylase inhibition assay was performed by the procedure of Keharom and co-workers (Keharom *et al.,* 2016). Pancreatic α-amylase was purchased from Sigma–Aldrich.

The dry mass extracts from 10 g of leaves or flowers were used to prepare different concentrations ranging from 40 μg/mL to 140 μg/mL. Luteolin 7-glucoside (1) and apigenin 7-glucoside (2) were tested at concentrations ranging from 0.25 μg/mL to 15 μg/mL.

The starch solution (1%) used as a substrate was prepared by boiling and stirring 0.5 g of starch in 50 mL of sodium phosphate buffer for 5 min. The pancreatic α-amylase solution was prepared by mixing 0.01 g of the enzyme in 10 mL of the sodium phosphate buffer. The color reagent was a solution containing 3,5-dinitrosalicylic acid (0.1 g), sodium potassium tartrate (2.99 g), sodium hydroxide (0.16 g) and the phosphate buffer (completed to 10 mL).

Twenty-five microliters of each plant extract and 25 μL of the enzyme were mixed in a 96-well plate and incubated for 10 min at 25°C. Then, 25 μL of the starch solution were added and the mixture was incubated under the same conditions. The reaction was stopped by adding 50 μL of a dinitrosalicylic color reagent. The mixture was incubated for 20 min at 85°C (Incubator CLN32STD POL-EKO Aparature, Poland). After cooling this mixture to room temperature, the absorbance was measured at 540 nm (Spectrophotometer Thermo Fisher SCIENTIFIC Multiskan Go, Finland). In the control, well dimethyl sulfoxide (DMSO, St. Louis, MO, USA) replaced the plant extract.

The blank sample in which the enzyme was replaced with the buffer solution was used to correct the absorption of the mixture. An acarbose solution at concentrations of 0.25 μg/mL to 1.0 μg/mL was used as a positive control. The inhibition percentage of α-amylase was assessed by the formula:

$$I_{\alpha\text{-amylase }\%}=100 \times (\Delta A_{control}-\Delta A_{sample})/\Delta A_{control}$$

$$\Delta A=A_{test}-A_{Blank}$$

Percent inhibition of the enzyme activity at the concentration range of 0.25–15 μg/mL (isolated flavoinods) and 40–140 μg/mL (extracts) were calculated and IC_{50} values were estimated by linear regression.

Cell culture and treatment. HepG2 (ATCC HB 8065) was purchased from American Type Culture Collection (Rockville, MD, USA). Liver cancer cells were maintained in Dulbecco's Modified Eagle's Medium (St. Louis, MO, USA) containing 10% fetal bovine serum (St. Louis, MO, USA) and antibiotics solution (St. Louis, MO, USA). The cells were grown in a humidified incubator at 37°C in the atmosphere of 5% CO_2. To assess the effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2), methanolic and ethyl acetate extracts of *S. pratensis* flowers and leaves on the measured parameters, $5x10^5$ cells were seeded per 100 mm culture dish. After 24 hours of initial incubation, the cells were treated with 2.5 μg/mL, 5 μg/mL and 10 μg/mL luteolin 7-glucoside (1), apigenin 7-glucoside (2), methanolic and ethyl acetate extracts of *S. pratensis* flowers (SK1, SK2) and leaves (SL1, SL2) or 0.1% vehicle DMSO as a control. Incubation was continued for subsequent 24 hours and then cells were harvested.

Viability assay. The effect of studied compounds on cell viability was assessed by the MTT assay, according to standard protocols described previously (Krajka-Kuźniak *et al.,* 2013). Briefly, 10^4 HepG2 cells were seeded per well in a 96-well plate. After 24 hours of preincubation in complete medium, flavonoids or extracts were added to the culture medium at various concentrations, and cells were incubated for subsequent 24 hours. DMSO concentration did not exceed 0.1%. After 24 hours, cells were washed twice with warm PBS buffer and fresh medium containing the MTT salt (0.5 mg/mL) was added. After 4 hours of incubation, formazan crystals were dissolved in acidic isopropanol and absorbance was measured at 570 nm and 690 nm. All of the experiments were repeated three times.

Nuclear and cytosolic preparation. The cytosol and nuclear extracts from HepG2 were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, CA USA).

RNA isolation. The extraction of total RNA from cells was performed using GeneMatrix Universal RNA Purification Kit (EurX, Gdańsk, Poland) according to the manufacturer's instructions.

Quantitative PCR. Total RNA was subjected to reverse transcription using the RevertAid Kit (Fermentas, Burlington, Canada), followed by quantitative real-time PCR. For real-time analyses, the Max-

Table 1. Primers used in RT-PCR.

	Forward primer	Reverse primer
NF-κB p50	5′ATCATCCACCTTCATTCTCAA	5′AATCCTCCACCACATCTTCC
NF-κB p65	5′CGCCTGTCCTTTCTCATC	5′ACCTCAATGTCCTCTTTCTG
COX-2	5′CCTGTGCCTGATGATTGC	5′CAGCCCGTTGGTGAAAGC
PBGD	5′TCAGATAGCATACAAGAGACC	5′TGGAATGTTACGAGCAGTG
TBP	5′GGCACCACTCCACTGTATC	5′GGGATTATATTCGGCGTTTCG

Table 2. Spectral analysis results of luteolin 7-glucoside (1) and apigenin 7-glucoside (2) isolated from *S. pratensis* flowers.

Compound	Identification results
1	**UV λnm:** MeOH 250, 335; MeOH/NaOAc 252, 267sh, 360sh, 401; MeOH/NaOAc/H_3BO_3 258, 359; MeOH/NaOMe 385, 268, 300sh; MeOH/$AlCl_3$ 274, 297sh, 360, 429; MeOH/$AlCl_3$/HCl 382 **ESI-MS m/z:** 447 $(M-H)^-$ **^{1}H NMR** (400 MHz, MeOH–d4): δ 3.19–3.53 (m, sugar-H), 3.58 (d, *J*=10.0 Hz, H5″), 5.06 (d, *J*=8.0 Hz, H-1″), 6.49 (d, *J*=2.0 Hz, H6), 6.06 (s, H-3), 6.79 (m, H8), 6.90 (d, *J*=8.3 Hz, H-5′), 7.36-7.42 (m, H2′, H6′) **^{13}C NMR** (100 MHz, MeOH- d4): 164.8 (C-2), 98.20 (C-3), 184.0 (C-4), 162.9 (C-5), 98.0 (C-6), 166.8 (C-7), 96.1 (C-8), 158,9 (C-9), 101.6 (C-10), 123.4 (C-1′), 129.9 (C-2′), 114.2 (C-3′), 147.1 (4′), 114.3 (5′), 120.5 (6′), 101.6 (C-1″), 74.7 (C-2″), 75.3 (C-3″), 69.08 (C-4″), 77.8 (C-5″), 62.43 (C-6″)
2	**UV λnm:** MeOH 270, 280, 312; MeOH/NaOAc 273, 310, 368; MeOH/NaOAc/H_3BO_3 269, 309; MeOH/NaOMe 272, 310, 366; MeOH/$AlCl_3$ 273, 308, 354; MeOH/$AlCl_3$/HCl 273, 305, 353 **^{1}H NMR** (400 MHz, MeOH–d4): δ 3.12–4,25 (m, sugar-H), 5,0 (s, Hz, H-1″), 6.91 (d, *J*=2.0 Hz, H6), 6.66 (s, H3), 7.03 (s, H8), 6.90 (d, *J*=8.0 Hz, H-5′), 7.33 (m, H3′, H5′), 7.78–7.84 (m, H2′, H6′) **^{13}C NMR** (100 MHz, MeOH- d4): 164.6 (C-2), 103.9 (C-3), 184.1 (C-4), 147.2 (C-5), 99.5 (C-6), 150.9 (C-7), 96.0 (C-8), 147,0 (C-9), 105.6 (C-10), 120.8 (C-1′), 124.1(C-2′), 116.8 (C-3′), 162.7 (4′), 114.9 (5′), 124.0 (6′), 99.5 (C-1″), 73.6 (C-2″), 74.7 (C-3″), 69.5 (C-4″), 79.6 (C-5″), 62.6 (C-6″)

ima SYBR Green Kit (Fermentas, Burlington, Canada) and a BioRad Chromo4 thermal cycler were used. The protocol started with 5 min enzyme activation at 95°C, followed by 40 cycles of 95°C for 15 s, 56°C for 20 s and 72°C for 40 s, and final elongation at 72°C for 5 min. Melting curve analysis was used for product specificity verification. Estimation of expression of *TBP* (TATA box binding protein) and *PBGD* (porphobilinogen deaminase) was used for data normalization. Sequences of primers, obtained from oligo.pl (Warsaw, Poland), that were used for the analysis of *NF-κB p50, NF-κB p65, COX-2, TBP* and *PBGD* are listed in Table 1.

Western blot analysis. Nuclear extracts (NF-κB p50, NF-κB p65) or cytosolic proteins (NF-κBp 50, NF-κB p65, COX-2) (100 μg) were separated on 10% SDS-PAGE slab gels and proteins were transferred to nitrocellulose membranes. After blocking with 10% skimmed milk, proteins were probed with rabbit polyclonal NF-κB p50, rabbit polyclonal NF-κB p65, goat polyclonal COX-2, rabbit polyclonal β-actin, rabbit polyclonal lamin antibodies (Santa Cruz, CA, USA). Either β-actin or lamin was used as an internal control. The alkaline phosphatase-labeled anti-goat IgG, or anti-rabbit IgG were used as the secondary antibodies in the staining reaction. Bands were visualized with BioRad AP Conjugate Substrate Kit NBT/BCIP. The amount of immunoreactive product in each lane was determined using the Quantity One software (BioRad Laboratories, Hercules, CA, USA). Values were calculated as relative absorbance units (RQ) per mg protein.

Statistical analysis. The data obtained were expressed as a mean of three replicates and the significant difference was considered at $p<0.05$. Biological activity was analyzed using a simple linear regression, and the coefficient of determination (R^2) was calculated and the statistical analysis was performed using the STATISTICA10 software followed by the Student's *t*-test.

RESULTS

Chemical analysis

The applied protocol described above allowed for isolation of luteolin 7-glucoside (1) and apigenin 7-glucoside (2). The chemical structure of the compounds was determined on the basis of ^{1}H ^{13}C NMR, ESI-MS and UV-spectroscopy analysis (Table 2) and by comparison with the data from the literature (Giang *et al.,* 2004; Mabry *et al.,* 1970; Gohari *et al.,* 2011, Asgharia *et al.*, 2015; Peng *et al.,* 2016). For comparison, in further studies, extract containing the flavonoid fraction was applied.

The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts on the α-amylase activity

The isolated flavonoids, and methanolic and ethyl acetate extracts from *S. pratensis* leaves (SL1, SL2) and flowers (SK1, SK2) were examined for their α-amylase inhibitory activity. The results are presented in Table 3. Luteolin 7-glucoside (1) showed stronger activity against α-amylase than apigenin 7-glucoside (2). Both glucosides (1, 2) had a moderate inhibitory effect on α-amylase, with the IC_{50} value of 13.2 μg/mL and 26.1 μg/mL, respectively, while the IC_{50} value for acarbose was 0.69 μg/mL. High inhibitory potency was demonstrated for the methanolic extract from *S. pratensis* leaves (SL1), with the IC_{50} value of 88.5 μg/mL. A similar activity was shown by the ethyl acetate extracts from the leaves (SL2) and methanol from the flowers (SK1) (IC_{50}=120.0 μg/mL and 119.1 μg/mL, respectively). The ethyl acetate extract from the flowers (SK2) showed the weakest activity (IC_{50}=211.3 μg/mL).

Table 3. α-Amylase inhibitory activities and IC_{50} values of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts.

C (μg/mL)	Inhibitory percentage (±S.D.)				C (μg/mL)	Inhibitory percentage (±S.D.)		
	SL1	SL2	SK1	SK2		1	2	Acarbose
40	37.16±0.17	32.62±0.29	20.57±0.14	19.27±0.31	0.25	9.54±0.23	5.21±0.10	31.44±0.14
80	36.66±0.37	36.33±0.33	29.52±0.28	27.66±0.28	0.5			45.74±0.21
100	48.14±0.30	48.14±0.22	33.78±0.07	30.70±0.18	1.0	10.75±0.11	8.64±0.23	61.05±0.17
120	51.61±0.07	51.61±0.25	45.61±0.09	33.21±0.24	1.5			81.11±0.11
140	53.41±0.30	53.47±0.15	69.00±0.37	37.43±0.02	2.0	12.15±0.13	11.15±0.47	
					5.0	20.32±0.27	16.19±0.15	
					10.0	43.66±0.43	24.72±0.27	
					15.0	54.15±0.12	30.35±0.22	
IC_{50}	88.52	119.97	119.10	211.28		13.23	26.09	0.69

The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and methanolic and ethyl acetate extracts from *S. pratensis* leaves and flowers on the HepG2 cells' viability

The MTT test was used to evaluate the effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts on cell viability. Within the concentration range of 0.1–50 μg/mL the tested compounds and extracts reduced the viability of the HepG2 cells in a dose-dependent manner (Fig. 1). Luteolin and apigenin 7-glucosides were more cytotoxic than the extracts.

On the basis of the above results of the MTT test, in further studies, the tested flavonoids and *S. pratensis* extracts were used at the concentrations of 2.5 μg/mL, 5 μg/mL and 10 μg/mL.

The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts on NF-κB activation, and NF-κB and COX-2 expression

Activation of NF-κB was evaluated in terms of translocation of its active subunits from cytosol into the nucleus. As is shown in Fig. 2, luteolin and apigenin 7-glucosides decreased the nuclear level of p50 and p65 subunits by about 21–23%, at concentration of 10 μg/mL. Ethyl acetate extract from leaves (SL2), had significantly diminished the nuclear level of NF-κB p50 and the cytosolic level of COX-2, by about 29% and 21%, respectively.

The effect of isolated luteolin, apigenin 7-glucosides and *S. pratensis* extracts on *NF-κB* and *COX-2* mRNA levels in HepG2 cells is shown in Table 4. At the concentration of 10 μg/mL, both glucosides and all studied extracts reduced the mRNA of *p65* subunit level by about 23–36% in comparison to the result obtained in the control group. A similar effect was observed for expression of *NF-κB p50*. Moreover, SK1 and SK2 diminished the level of *NF-κB p50* mRNA at a concentration of 5 μg/mL. At the same concentration, SL1 and apigenin 7-glucoside had decreased the transcript level of *NF-κB p65*. Expression of *COX-2* was decreased after incubation with both glucosides and extracts, but the difference was not statistically significant.

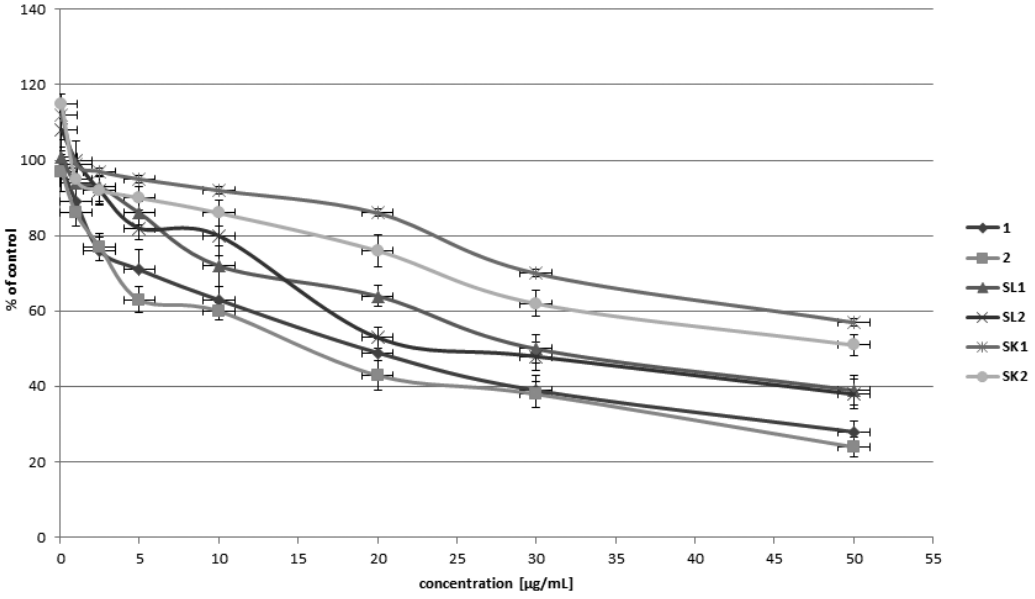

Figure 1. The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and S. pratensis extracts on the HepG2 cell line viability. Data are expressed as means ± S.E.M. from three separate experiments.

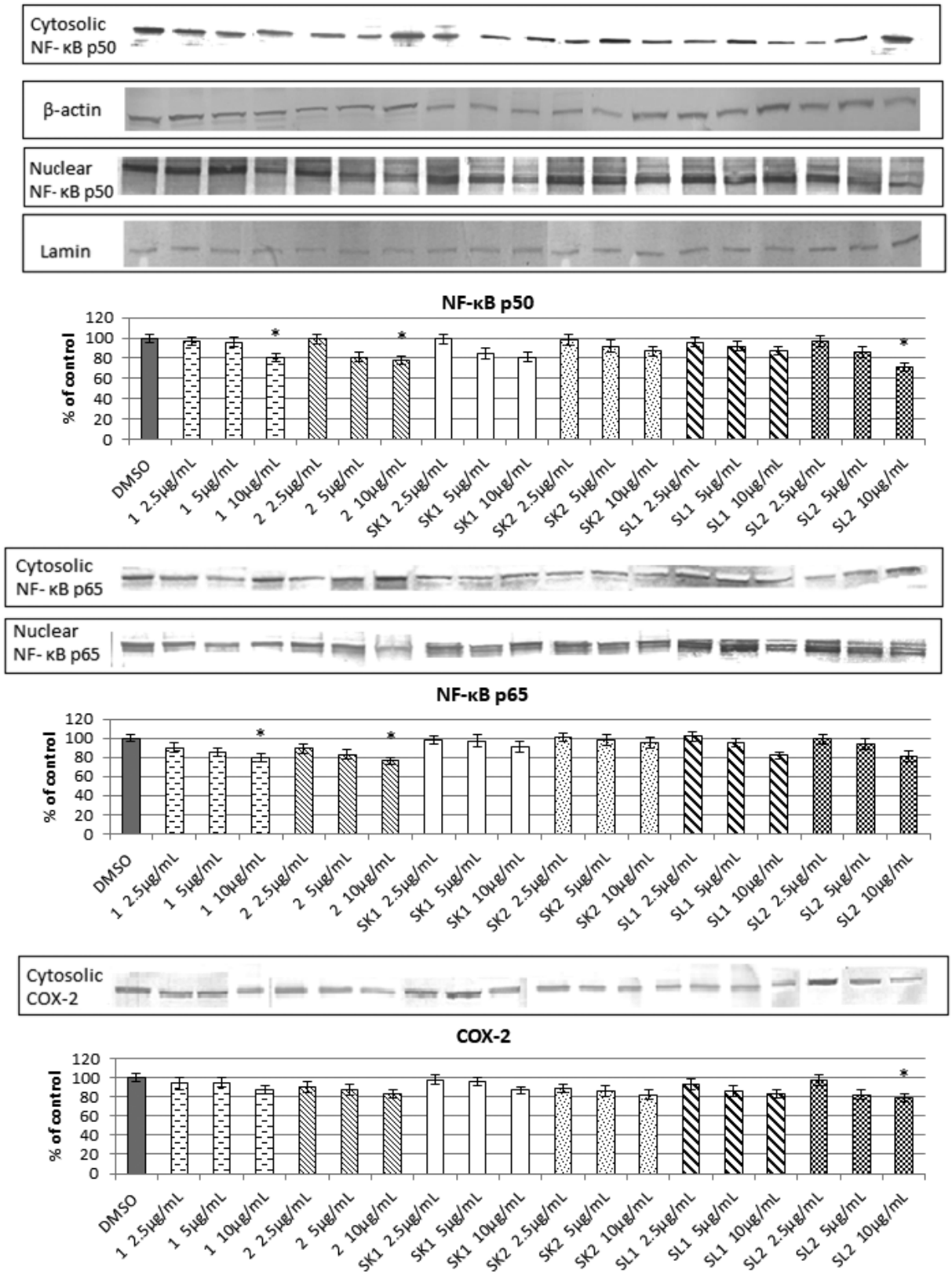

Figure 2. The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts on NF-κB and COX-2 protein level in HepG2 cells.
Data (means ± S.E.M.) of Western blot analysis of the cytosolic and the nuclear content of p50 and p65, and the cytosolic content of COX-2 from three separate experiments; representative immunoblots are shown. The sequence of the bands corresponds to the sequence of bars in the graph. The results of Western blot analysis are compared with the control level, which equals 100%. The asterisk (*) above the bar denotes a statistically significant difference from the control group, $p<0.05$.

Table 4. The effect of luteolin 7-glucoside (1), apigenin 7-glucoside (2) and *S. pratensis* extracts on expression of the NF-κB p50 (a), NF-κB p65 (b) and COX-2 (c) in HepG2 cells.
Data are means ± S.E.M. from three separate experiments. The values were calculated as mRNA level in comparison to control cells (expression equals 1). The asterisk (*) denotes a statistically significant difference from the control group, $p<0.05$.

a

C (μg/mL)	The level of the *NF-κB p50* transcript in comparison to control cells (±S.E.M.)					
	SL1	SL2	SK1	SK2	1	2
2.5	0.94±0.07	0.94±0.11	0.82±0.09	0.88±0.12	0.90±0.10	1.08±0.10
5	0.87±0.08	0.80±0.10	0.79±0.11*	0.74±0.07*	0.86±0.09	1.05±0.09
10	0.78±0.07*	0.79±0.06*	0.63±0.09*	0.62±0.07*	0.79±0.1*	0.80±0.10

b

C (μg/mL)	The level of the *NF-κB p65* transcript in comparison to control cells (±S.E.M.)					
	SL1	SL2	SK1	SK2	1	2
2.5	0.89±0.08	0.97±0.12	0.91±0.09	0.94±0.09	0.89±0.11	0.81±0.12
5	0.77±0.09*	0.84±0.14	0.89±0.11	0.87±0.08	0.81±0.09	0.74±0.07*
10	0.64±0.09*	0.76±0.06*	0.73±012*	0.77±0.07*	0.72±0.1*	0.67±0.06*

c

C (μg/mL)	The level of the *COX-2* transcript in comparison to control cells (±S.E.M.)					
	SL1	SL2	SK1	SK2	1	2
2.5	0.96±0.13	0.95±0.13	0.94±0.08	0.99±0.11	0.98±0.09	0.95±0.12
5	0.89±0.10	0.88±0.19	0.87±0.11	0.92±0.11	0.91±0.10	0.88±0.10
10	0.83±0.11	0.80±0.11	0.81±0.12	0.87±0.10	0.83±0.09	0.82±0.11

DISCUSSION

Chemical composition of the *S. pratensis* herb was not well described so far. Our earlier study showed a substantial amount of polyphenolic compounds in the leaves' extracts from this plant (Witkowska-Banaszczak & Długaszewska, 2017). Thus, the aim of this study was further characterization of these components and evaluation of their biological activity. Two flavonoid glucosides, luteolin and apigenin were isolated. Their effect on the α-amylase activity and NF-κB activation was compared with that exerted by the extracts containing the whole flavonoid fraction obtained from flowers or plant leaves.

The results of our study confirmed inhibition of the α-amylase activity by luteolin and apigenin 7-glucosides (Kim *et al.*, 2000; Funke & Melzing, 2006; Li *et al.*, 2018). Moreover, luteolin 7-glucoside isolated from *S. pratensis* showed a stronger inhibitory effect on α-amylase activity than apigenin 7-glucoside. Since the biological activity of flavonoids depends on the number of hydroxyl residues in the core molecule, it may be concluded that the presence of an additional OH group in the B ring of luteolin 7-glucoside makes this compound a more potent inhibitor of α-amylase. These studies have confirmed earlier reports about the influence of hydroxyl substituents and sugar molecules in the flavonoids on inhibition of α-amylase (Tadera *et al.*, 2006; Sales *et al.*, 2012; Asgharia *et al.*, 2015).

Comparison of the effect of isolated luteolin and apigenin 7-glucosides with herb extracts indicated a stronger inhibitory potential of *S. pratensis* leaves' extract. A high content of flavonoids in this preparation (1.18%) in comparison with the flowers extract (0.23%) may be responsible for this effect (Witkowska-Banaszczak & Długaszewska, 2017).

The results of this study also indicated that the methanolic extracts are more efficient than the ethyl acetate in α-amylase activity inhibition. Thus, such preparation should be recommended for further studies. Additional research into the chemical profile of the extracts will probably allow determining the compounds or groups of compounds affecting amylase inhibition.

α-Amylases catalyze the specific cleavage of α-1,4 glycosidic bonds in polysaccharides, such as starch and glycogen. Human α-amylase is one of the major secretory products of the pancreas (P-amylase) and salivary glands (S-amylases). Human S- and P-amylases are encoded by AMY-1 and AMY-2A genes, respectively. The AMY-2B gene encodes α -amylases with a very similar amino acid sequence to the S- and P-amylases (Yokouchi *et al.*, 1990), which are expressed in the lung carcinoid tissue (Doi *et al.*, 1991; Tomita *et al.*, 1989). It was shown that human liver amylase is encoded by the same gene. Thus, this α-amylase isoform may be related to hepatocellular carcinoma (HCC) (Koyama *et al.*, 2001). Chronic inflammation plays an important role in pathogenesis of HCC. The key element of this process is activation of NF-κB (Hwang *et al.*, 2011).

Thus, in this study we also evaluated the effect of luteolin and apigenin 7-glucosides, and methanolic and ethyl acetate extracts from *S. pratensis* leaves and flowers on the NF-κB transcription factor activation and expression. Luteolin and apigenin 7-glucosides decreased expression of *NF-κB* and *COX-2*. The expression of the latter is controlled by NF-κB. A similar effect was observed after treatment of HepG2 cells with extracts from *S. pratensis* leaves and flowers. However, activation of NF-κB evaluated in terms of translocation of its active subunits from the cytosolic to the nuclear fraction was significantly affected only by isolated flavonoids and ethyl acetate extracts from *S. pratensis* leaves. Thus, the effect of *S. pratensis* preparation on NF-κB signaling was moderate.

However, luteolin 7-glucoside isolated from this herb seems be the most potent inhibitor of α-amylase activity, as well as of NF-κB p50 and NF-κB p65. Our re-

sults confirmed the observation made by other authors on luteolin potential as an anti-inflammatory agent acting through downregulation of NF-κB and subsequently downregulation of *COX-2* expression.

In this regard, Xue and others (Xue *et al.,* 2017) showed that luteolin and apigenin downregulate *COX-2* expression in HepG2 cells. Similar observations in the case of luteolin appeared in the paper of Hwang and others (Hwang *et al.,* 2011). They showed that luteolin inhibits NF-κB signaling pathways in HepG2 cells. Additionally, other studies using pancreatic carcinoma cells (PANC-1, CoLo-357 and BxPC-3 cells) confirmed the inhibitory effect of luteolin on NF-κB (Cai *et al.,* 2012).

Overall, the results of this study indicate that *S. pratensis* flavonoids may modulate the metabolic and signaling pathways whose deregulation is related to pathogenesis of liver cancer. Further studies are required to confirm these observations and assess the chemopreventive and/or therapeutic potential of the *S. pratensis* herb.

Conflicts of Interest

Authors declare that they have no conflict of interest to disclose.

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