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Biological activity of plant extract isolated from *Papaver rhoeas* on human lymfoblastoid cell line

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Varied medicinal plants are known as a source of natural phytochemicals with antioxidant activities that can protect organisms from oxidative stress and from various chronic diseases. *Papaver rhoeas* has a long history of medicinal usage, especially for ailments in adults and children. The possible cytotoxicity, genotoxicity and potential antioxidant effect of plant extract isolated from flowers of *Papaver rhoeas* was investigated in human lymfoblastoid cell line (TK6). Antioxidant activity of this extract was determined using the DPPH assay. The plant extract exhibited dose dependent free radical scavenging ability. The growth activity assay was used for determination of cytotoxicity. To assess potential genotoxicity the comet assay was used. The lower extract concentrations (0.25 and 0.5 mg/ml) neither exerted cytotoxic, nor genotoxic effects in TK6 cells but they stimulated cell proliferation. The concentration 25 mg/ml scavenged almost 85% of DPPH free radical. On the other hand, this concentration had strong cytotoxic and genotoxic effect on TK6 cells.

The balance between beneficial and harmful effects should be always considered when choosing the effective dose.

Key words: antioxidant activity; DPPH radical; Papaver rhoeas, cytotoxicity, genotoxicity, comet assay

Plants are universally recognized as a vital part of the world's natural heritage and up to 80% of the population relies on plants for their contribution to a primary healthcare. The use of plants in traditional medicine is widespread. Many plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. It is commonly accepted that due to oxidative stress, reactive oxygen species (ROS) such as superoxid, hydroxyl and peroxyl radicals are generated [1]. Recently much attention has been given to the natural phytochemicals with antioxidant, antimutagenic and anticarcinogenic activities [2-8]. These compounds are of great importance for chemoprevention and potential cancer biotherapy.

Papaveraceae is a family of flowering plants. The flowers of poppy are chiefly employed as a mild pain reliever and as a treatment for irritable coughs; it also helps to reduce nervous over-activity [9]. The flowers and petals are anodyne, expectorant, hypnotic, slightly narcotic and sedative. An infusion is taken internally in the treatment of bronchial complaints and coughs, insomnia, poor digestion, nervous digestive disorders and minor painful conditions. The flowers are also used in the treatment of jaundice and – in very small quantities– as a sleep-inducing drug [9, 10].

Papaver rhoeas plant extract contains a lot of flavonoids: kaempferol, quercetin, luteolin and hypolaetin and glycosides 3-O-β-D-glucopyranosylquercetin (isoquercetin), 3-O-β-D-glucopyranosyl kaempferol (astragaline), 3-O-β-D-galactopyranosylkaempferol (hyperoside) [11]. Antioxidant activity of the kaempferol [12-13], quercetin [14-15] and luteolin [16] was already demonstrated. Total phenolic compounds (9.73 – 19.91 mg GAE/g) and total flavonoids (7.904-11.45 mg QE/g) were determined in fresh petals [17].

Besides flavonoids and their glycosides *Papaveraceae* family are known for the presence of alkaloids as morphine, codeine, narcotine, thebaine and papaverine [8, 18, 19].

The aim of this study was to determine biological activities of the methanolic extract isolated from *Papaver rhoeas*. Its antioxidant activity was studied by the DPPH (2,2-diphenyl-2-picrylhydrazyl) radical scavenging assay. The cytotoxic and genotoxic effects of this extract were assessed using – the proliferating (growth) activity assay and the comet assay.

Materials and methods

Plant material. The plant *Papaver rhoeas* isolated from flowers was harvested from the area of middle part of Slovakia, Banska Bystrica. The separated plant material was air-dried to dryness at a room temperature, cut into small pieces and then extracted with methanol at 65°C. This procedure was repeated 5 -times. The extract was then filtered and concentrated *in vacuo* and the rest of the water was removed by azeotropic destilation with benzene. The final extracts were kept in the dark at +4 °C until tested.

Antioxidant activity assessment. Antioxidant activity was measured in terms of the DPPH radical scavenging ability with slight modifications [20]. L-ascorbic acid was used as a reference sample (as a standard). The hydrogen atoms or electron-donation ability of the corresponding extracts and some pure compounds were measured from the bleaching of a purple-coloured methanol solution of DPPH [21]. To an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl, test extracts at different concentrations were added. The reaction mixtures were shaken vigorously and then kept in the dark for 20 min [1]. The absorbance of the resulting solutions was measured by spectrophotometer (SPECTRONIC, GENESYS 10 Bio, ELECTRON CORPORATION, Pragolab, Bratislava) at 517 nm, against blank. Decrease of DPPH solution absorbance indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated according to formula:

$I\% = (A_b - A_s/A_b) \times 100.$

A_b – the absorbance of the control reaction (containing all reagents except the test compound)

A_s – the absorbance of the test compound

The IC_{50} (concentration causing 50 % inhibition) value of extract was determined. Tests were carried out three-times.

Treatments. The human lymfoblastoid TK6 cells were cultured in RPMI medium supplemented with 10 % fetal calf serum (heat up first time on 50 °C) and 1% of antibiotics (penicillin/streptomycin). Cells were cultivated in suspension in tissue cell flask at 37 °C in humidified atmosphere.

Exponentially growing cells were treated for 24 h with various concentrations of plant extract or 100 μmol/l of hydrogen peroxide (H_2O_2) as a positive control. Control cells were kept in a fresh culture medium. After the treatment, the cells were centrifuged (900 RPM, 5 minutes, 4°C), washed with PBS, resuspended in the cell culture medium and divided into two portions: for evaluation of the proliferation activity and for the comet assay.

The proliferating (growth) activity assay. After the 24 h of treatment with plant extract, TK6 cells were counted and pipetted (2 x 10^4 cells) to each well with a growth medium. At 24 h intervals for 5 days the cells were counted for each concentration of plant extract and for the control, as well. Each measurement was repeated three times.

The comet assay. The comet assay was performed following the protocol of Singh *et al*., 1988 with modification [22].

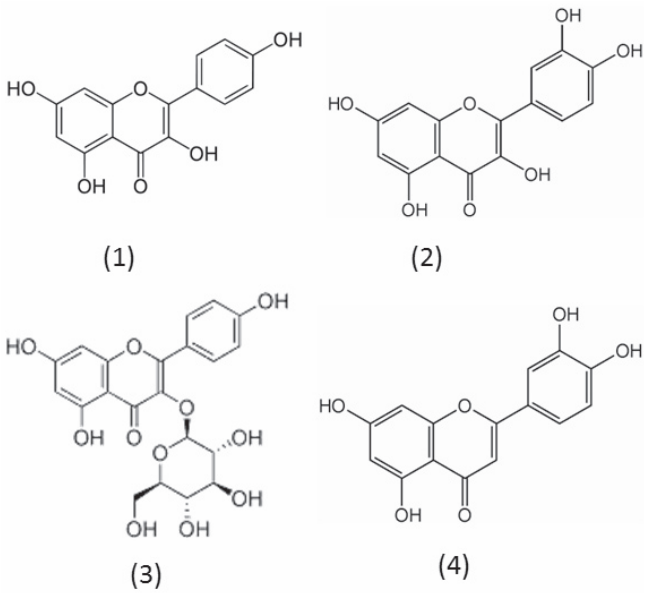

Figure 1. Flavonoids present in *Papaver rhoeas*: **(1) kaempferol, (2) quercetin, (3) astragaline, (4) luteolin**

Microscope slides were pre-coated with a thin layer of 1 % normal melting point agarose. Cells (1 x 10^4 per gel) were resuspended in 1 % low-melting agarose and spread on a base layer on a microscope slide. Two gels per slide were prepared. The cells were lysed in lysing solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris–HCl, 1 % Triton X-100, pH 10) for 1 h at 4°C to remove cellular proteins. After lysis slides were immersed for 20 min in an electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) at 4°C for DNA unwinding before electrophoresis at 25 V and 300 mA for 30 min. The slides were removed, neutralised with PBS (0.4 mM, pH 7.5) and cold water and stained with 10 μl DAPI (1 μg/ml). DAPI – stained nucleoids were evaluated with the Leica DMI 6000 B fluorescence microscope. For each sample, 100 comets were scored by computerised image analysis and CCD camera and computer software Comet assay IV, Perceptive instruments Ltd. UK) determining the percentage of DNA in the tail which is linearly related to the frequency of DNA breaks. 2 gels per slide and dose were used in each experiment. Three independent experiments each with two parallels were performed.

Results

Five concentrations (0.25 – 25 mg/ml) of the methanolic extract isolated from *Papaver rhoeas* were investigated for its possible antioxidative capacity by the DPPH free radical-scavenging assay (Fig. 2a). The extract exhibited scavenging activity in a dose response relationship. The results were compared with that of ascorbic acid, which was used as the positive control due to a high scavenging activity (Fig 2b). The methanol extract of *P. rhoeas* was able to reduce the stable free radical

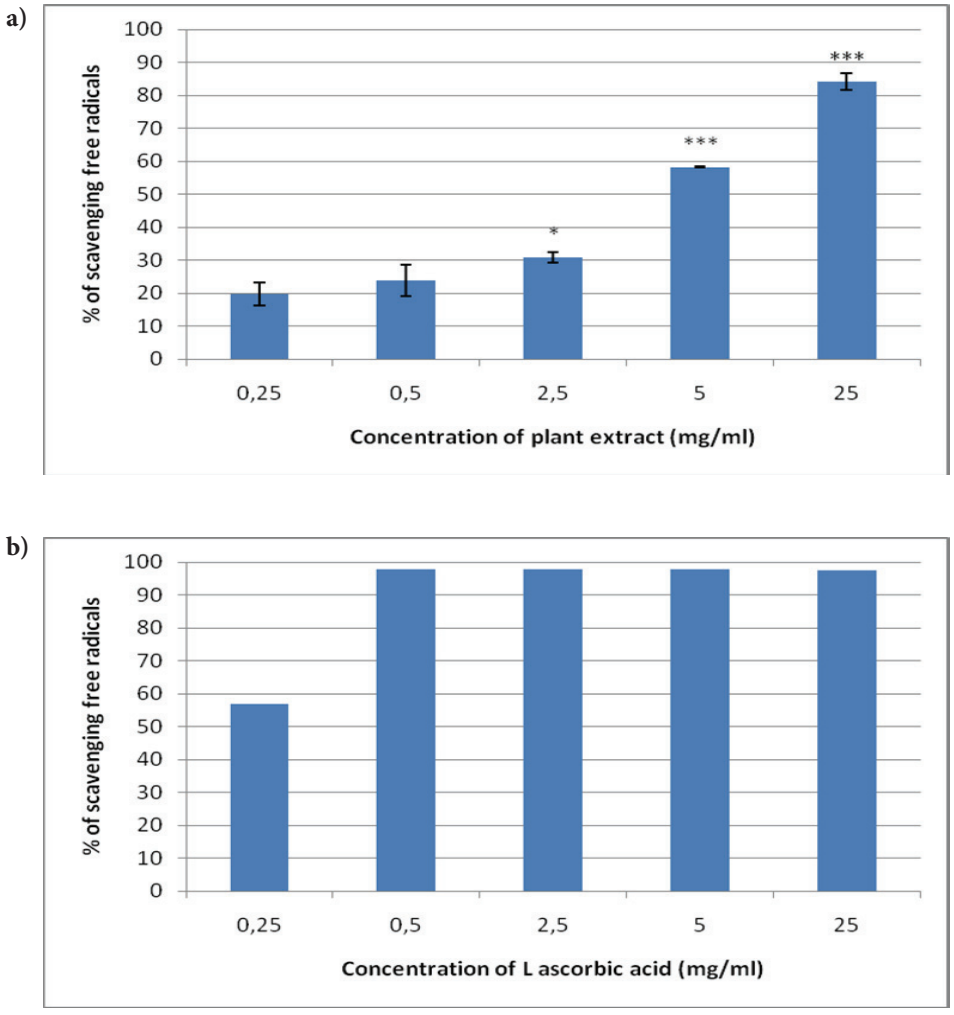

Figure 2. a) Antioxidant activity of methanolic extract isolated from flowers of *P. rhoeas*. Different concentrations of plant extract were studied (0.25-25 mg/ml) and IC_{50}=1.4 ± 0.525 mg/ml was determined. All experiments were performed independently at least three times with two parallels. Mean values ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. b) Antioxidant activity of L-Ascorbic acid, which was used as a reference. IC_{50}=5.918 ± 0.4257 µg/ml.

2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow-coloured DPPH with an IC_{50} of 1.4 ± 0.525 mg/ml. IC_{50} of ascorbic acid were 5.918 ± 0.4257 µg/ml.

Cytotoxicity of plant extract in TK 6 cells was measured by the proliferation assay (Fig.3, Table 1). Cell growth is the change in the number of cells in the population over time, and is used to determine population doubling time. The highest inhibition of cells growth was observed at the concentrations 5 mg/ml and 25 mg/ml after the treatment with plant extract. However, lower concentrations did not cause inhibition of cell proliferation. Two lowest concentrations (0.25 and 0.5 mg/ml) even stimulated cell growth.

DNA strand breaks were measured after the 24 h of TK6 treatement with different concentrations of plant extract (from 0.25 to 25 mg/ml) using the alkaline comet assay (Fig. 4). The concentration 2.5, 5 and 25 mg/ml induced DNA strand breaks. H_2O_2, a strong oxidant used as a positive control, induced a significant increase of DNA strand breaks (Fig. 4).

Discusion

There are several possibilities of biochemical events causing antioxidation [23]. It could be a result of metal chelation [24], scavenging of free radicals [25], enzyme inhibition [26], and induction of the protective enzymes expression [27]. In this study we proved that the methanol extract of *P. rhoeas* was able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) with an IC_{50} of 1.4 ± 0.525 mg/ml (Fig. 2a) and exhibited scavenging activity. The mechanism of the reaction between antioxidant and DPPH radical depends on the structural conformation of the antioxidant. Some compounds react very quickly with DPPH, reducing a number of DPPH molecules equal to the

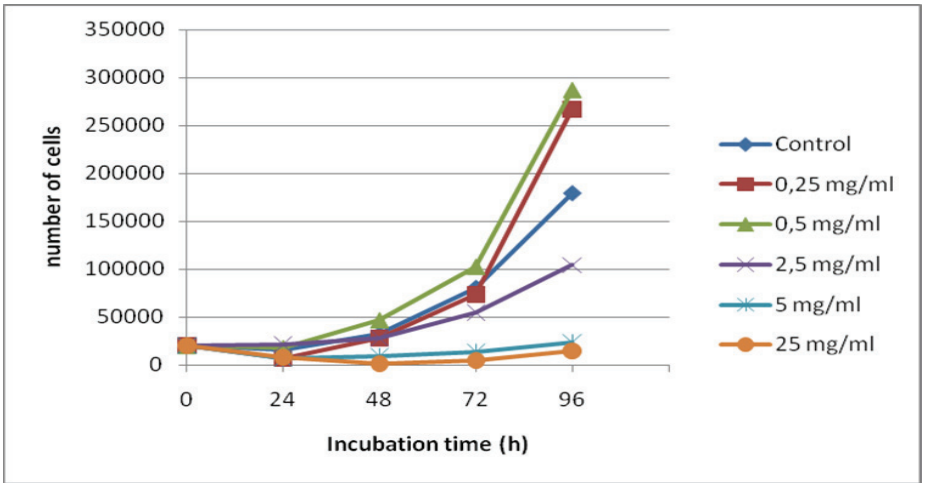

Figure 3. Growth curve (cell proliferation) of TK6 cells monitored after the 24 h of treatment in 0, 24, 48, 72 and 96 h incubation intervals with various concentrations of methanolic plant extract from *Papaver rhoeas* (0.25-25 mg/ml).

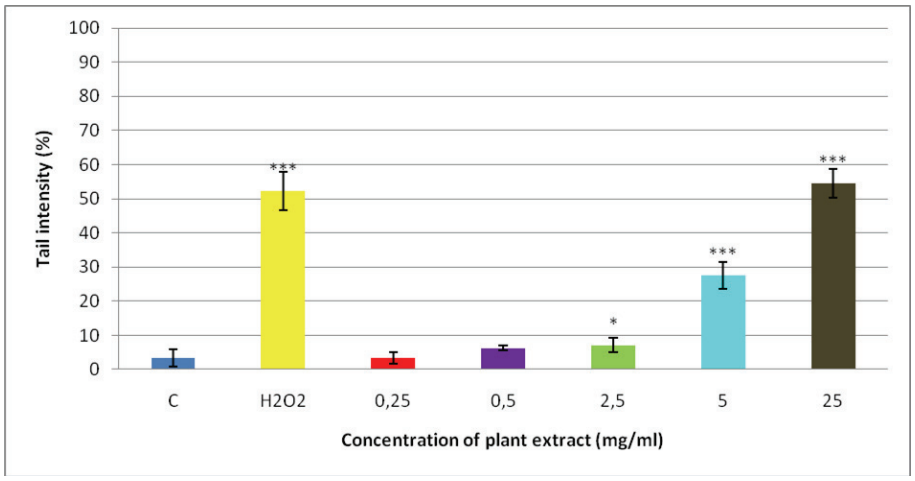

Figure 4. DNA damage of TK6 cells treated with plant extract or H_2O_2. TK6 cells were incubated for 24 h at 37 °C with different concentrations of plant extract (0.25-25 mg/ml) and H_2O_2 (100 µM). Mean values ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 1. Cytotoxicity effect of *Papaver rhoeas* extract. Proliferation of TK6 cells was measured each 24 h incubation intervals. All experiments were performed independently at least three times with two parallels. Mean values of number of cells ± SD. ***$P < 0.001$.

Concentration of plant extract (mg/ml)	Number of TK6 cells ± SD				
	0 h	24 h	48 h	72 h	96 h
0	20000	15463.33 ± 588.88	32165.83 ± 629.28	80366.67 ± 2113.449	179133.3 ± 1443.145
0.25	20000	6600 ± 303.315 ***	27816.67 ± 392.003 ***	73450 ± 564.8 ***	266816.7 ± 1919.966 ***
0.5	20000	17800 ± 485.798 ***	46816.67 ± 1268.726 ***	102400 ± 3618.839 ***	286583.3 ± 2444.959 ***
2.5	20000	21133.33 ± 970.91 ***	27950 ± 692.098 ***	54483.33 ± 2936.267 ***	104266.7 ± 1319.567 ***
5	20000	6683.33 ± 354.49 ***	8800 ± 414.729 ***	13283.333 ± 563.619 ***	23333.33 ± 496.65 ***
25	20000	7766.67 ± 294.39 ***	1533.33 ± 801.664 ***	4466.67 ± 476.095 ***	14300 ± 1466.97 ***

number of the hydroxyl groups [21]. The free radical-scavenging activity of flavonoids is dependent on the presence of OH groups, especially 3-OH. Flavonoids with a 3-OH and 3′,4′-cathecol are reported to be 10-fold more potent than flavonoids without this groups in structure [28]. Plant extracts isolated *from Chelidonium majus*, *Cynnara cardunculus*, *Teucrunium ramosissimum* etc. are reported to have scavenging effect and flavonoids contribute to this effect [4, 7, 29]. Our results showed that *Papaver rhoeas* extract has antioxidant activity similarly mediated by the scavenging activity of flavonoids.

Potential cytotoxicity and genotoxicity of plant extract isolated from *Papaver rhoeas* was investigated on the human lymfoblastoid cell line. Interestingly, the concentration 25 mg/ml that scavenged almost 85% of DPPH free radical had strong cytotoxic and genotoxic effects on TK6 cell line. Our results show that high antioxidant capacity does not necessary mean lack of cytototoxicity or genotoxicity. Hudecova et al. (2010) also found cytotoxic and genotoxic effects of plant extract isolated from *Gentiana asclepiadea* applied at high concentrations on mammalian (monkey) COS1 cells, despite these concentrations exhibited antioxidative activities [30].

Several beneficial properties have been attributed to flavonoids as dietary compounds, including antioxidant, anti-inflammatory, and anticarcinogenic effects. However, flavonoids have yet to pass controlled clinical trials for efficacy, and their potential for toxicity is an understudied field of research [31]. These findings indicate that there is no correlation between antioxidant effect and cyto- and genotoxicity of plant extract. There are some studies, where the toxicity of flavonoids was confirmed. A large clinical study by Knekt et al, in which 9959 men and women were followed for 24 y, showed an inverse relation between the intake of flavonoids (quercetin) and lung cancer. It was shown, that several flavonoids are toxic for human cells [32]. Moreover, it should be stressed that *Papaveraceae* family is known for presence of alkaloids, and the toxic effect of alkaloids is known. There are studies, where alkaloids were cytotoxic on human cells [33]. Papaverine, characteristic alklaloid in *Papaveraceae* family has cytotoxic effet on human cells [34].

In our study we observed cytotoxic effect, an inhibition of cell proliferation. Proliferation activity starts with a lag, growing phase and reaching a saturation density. The cell population's lag phase is the time for the cell to recover from subculture; the log phase, in which the cell number increases exponentially; and a plateau phase, in which the culture becomes confluent or dense and the growth rate slows or stops. An increase in cell number is also a frequently used method of assessing the effect of hormones, nutrients, and so forth on a specific cell type. Growth, or increase in total cell number over time, is a good measure of a biological response because it is so broadly defined and influenced by many different factors, including mitogens, changes in nutrient level, transport, membrane integrity, attachment factors (for cells growing attached on the surface) [35]. Proliferation activity curve (Fig. 3, Table 1) shows the cytotoxic effect of *P. rhoeas* extract on TK6 cells in the highest concentrations 5 and 25 mg/ml used. Interestingly, lower concentrations (0.25 and 0.5 mg/ml) did not cause inhibition of cell growth but have stimulating effect on TK6 cells proliferation.

Measurement of DNA single strand breaks in TK6 treated for 24 h with different concentrations of plant extract (from 0.25 to 25 mg/ml) was performed by the comet assay. The low doses which showed stimulating effect on cell proliferation were found to be non-genotoxic as they did not induce DNA breaks. The concentration 25, 5 and 2.5 mg/ml induced DNA strand breaks probably due to presence of alkaloids. Their genotoxic effect on human cells was documented earlier [33].

To conclude, our study shows that plant extract isolated from *Papaver rhoeas* possess a high antioxidant activity, but the balance between beneficial and harmful effects should be always considered when choosing the effective dose.

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