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COMPOSITIONS OF POLYPHENOLS IN WILD CHIVE, MEADOW SALSIFY, GARDEN SORREL AND AG YONCHA AND THEIR ANTI-PROLIFERATIVE EFFECT

D. Moravčíková, Z. Kuceková, J. Mlček, O. Rop, P. Humpolíček

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Abstract

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During past decades a lot of experimental studies shown that polyphenols have anti-carcinogenic properties have been published. Their antioxidant and tumour arresting effects have been demonstrated using both *in vitro* and *in vivo* studies many times. Aim of the present study was to investigate the content of polyphenols in edible flowers of Ag yoncha, Wild chive, Meadow salsify and Garden sorrel and their effect on proliferation activity of human hepatocellular carcinoma cells which has not been studied yet. Anti-proliferative effect was evaluated *in vitro* using following concentrations of polyphenols 100, 75, 50 and 25µg/ml. Also, phenolic composition was determined by the high performance liquid chromatography. The present results indicate that even low concentrations of edible flowers' polyphenols inhibited cell proliferation significantly. This effect was observed at all studied edible flowers extracts and was independent on the compositions of individual polyphenols. Results indicate possible employment of studied edible flowers for prevention as well as for treatment of cancers.

proliferation, Ag yoncha, Wild chive, Meadow salsify, Garden sorrel, polyphenols, HepG2

A lot of published studies prove that connection between polyphenols and one of the most common disease – cancer exists (Araújo *et al*., 2011; Saunders and Wallace, 2010; Milbury, 2009). Polyphenols (PF) are phytochemicals (Link *et al*., 2010) contained in green tea (Johnson *et al*., 2010; Luceri *et al*., 2002; Kuroda *et al*., 1999) or fruits (Iwasawa *et al*., 2011; Mahdavi *et al*., 2010; Manson, 2003; Rop *et al*., 2011; Milbury, 2009) for example. Every plant contains different polyphenol composition (Kuroda *et al*., 1999). Due to the fact that PF have many functions in organism such as free radical-scavenging or metal chelation and enzyme modulation (Rodrigo *et al*., 2011), they can act as antioxidants (Schlachterman *et al*., 2008), anti-angiogenics (Oak *et al*., 2005), selective estrogen receptor (ER) modifiers (Harris *et al*., 2005; Damianaki *et al*., 2000), anti-carcinogenic and anti-inflammatory agents (Roussou et al., 2004). Polyphenols possess anti-cancer properties and they interfere with cancer iniciation, promotion and progression (Link *et al*., 2010; Araújo *et al*., 2011). As another significant properties of polyphenols we can mention their inhibitory action against nitrosation reactions (Kuroda *et al*., 1999), inhibition of cell proliferation-related activities (Soleas *et al*., 2002), induction of cell apoptosis (Lin *et al*., 1999) and cell cycle arrest (Nichenametla *et al*., 2006), blockade of mitotic signal transduction through modulation of growth factor receptor binding (Lin *et al*., 1999), nuclear oncogene expression (Link *et al*., 2010) and inhibition of DNA synthesis (Navarro-Peran *et al*., 2007).

The aim of present study is to determine the effect of polyphenols occurring in the edible flowers

(Wild chive, Garden sorrel, Meadow salsify and Ag yoncha) on cell proliferation and to evaluate preventive effects of these polyphenols on hepatic cells.

MATERIAL AND METHODS

Extraction conditions

Polyphenols were extracted from flowers of Wild chive (*Allium schoenoprasum*), Garden sorrel (*Rumex acetosa*), Meadow salsify (*Tragopogon pratensis*) and Ag yoncha (*Trifolium repens*) as follows. Frozen edible flowers were homogenized in 90% methanol (2ml/g) and subsequently extracted at 4 °C for 30 minutes. After extraction the centrifugation at 1990 rpm was employed for 10 minutes to separate the supernatant and sediment was subjected to new extraction. This process was repeated three times. The supernatants containing polyphenols were dried using Laborota4011 digital (Heidolph, Germany). Finally, the extracts were concentrated to the concentration of 1000 mg/ml of polyphenols. PF were not separated from the other active substance.

Cell cultivation

The human hepatocellular carcinoma cell line (HepG2) from ATCC (HB-8065) was used. HepG2 cells were cultivated in ATCC-formulated Eagle's Minimum Essential Medium, (ATCC) added 10% fetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamycine (PAA Laboratories GmbH, Austria).

Anti-proliferation test

The samples of polyphenols were diluted in culture medium (DMEM) to obtain the solutions with concentrations of 100, 75, 50 and 25µg/ml. All dilutions were used up to 24 hrs. Cells were pre-cultivated for 24 hrs and the culture medium was subsequently replaced by dilutions. As a control experiment, pure extraction medium without polyphenols was used. To assess anti-proliferatic effect on HepG2 cells, the MTT assay (Invitrogen Corporation, USA) was performed after three-day cell cultivation in dilutions. The absorbance was measured at 540 nm by Sunrise microplate absorbance reader (Tecan, Switzerland). The cell viability, expressed as absolute value of cells present in respective dilution relatively to cells cultivated in pure dilution medium without polyphenols, is presented. All the tests were performed in quadruplicates. The morphology of cells was assessed after their cultivation in dilutions after 24 hrs. The cells from each culture plate were observed in an inverted Olympus phase contrast microscope (Olympus, CKX41). The differences between observed absorbance were detected by T-Test using Statistica for Windows.

Determination of polyphenols

A standard solution of tannin was prepared from 50 mg of tannin dissolved in water to volume of 100 ml. The standard solution of tannin was added using pipette to six 50 ml flasks in volumes 0.2, 0.3, 0.4, 0.5ml. One milliliter of extract was added to seven flasks and dissolved as needed. Twenty milliliters of distilled water and 1ml of the Folin-Ciocalteu reagent was added to every flask. After three minutes was added 5ml of 20% solution Na_2CO_3, the solutions were mixed and the distilled water was added to volume 50ml. After 30 minutes color intensity was measured at 700nm compared to control (no tannin).

Chromatography

Determination of individual polyphenols was carried out using the Dionex UltiMate 3000 high performance liquid chromatography (HPLC) system. For separation of polyphenols column Supelcosil LC-18-DB (25 cm × 4.6 mm I.D., S-5µm) was used. The extraction method described by Lee and Ong (2000) was used for the determination. The data presented are the average values calculated from three measurements.

RESULTS

We detected following polyphenol compounds by HPLC in this study. Edible flowers used in this study (Ag yoncha, Wild chive, Meadow salsify and Garden sorrel) contain: gallic acid, coumaric acid, ferulic acid, rutin, resveratrol, vanillic acid, sinapic acid, catechin, caffeic acid and quercentin. The content of individual polyphenols is presented in Tab. I. Ag Yoncha contains gallic acid (24.29µg/g), catechin (49.73µg/g), caffeic acid (30.84µg/g) and quercentin (143.00µg/g). Garden sorrel contains 4 kinds of polyphenols: resveratrol (10.09µg/g), vanillic acid (34.41µg/g), sinapic acid (1507.61µg/g) and catechin (19.93µg/g). The third herb Meadow salsify contains the most polyphenols from presented edible flowers. There were determinate 6 types of polyphenols as gallic acid (226.17µg/g), ferulic acid (33.19µg/g), rutin (15.01µg/g), resveratrol (2.34µg/g), sinapic acid (18.06µg/g) and caffeic acid (46.77µg/g). Last presented herb Wild chive contains gallic acid (29.88µg/g), coumaric acid (30.07µg/g), ferulic acid (131.43µg/g) and rutin (3.00µg/g).

Anti-proliferation activity was found in all studied edible flowers and in all tested concentrations. This effect is expressed by statistically significant differences between studied herbs and control (Tab. II.).

The Fig. 1 shows anti-proliferation effect of used polyphenols extracts on HepG2 cells. The lowest average values of anti-proliferation activity, expressed as absorbance of MTT assay, in Ag Yoncha were detected in concentrations 50 µg/ml (0.2841) and 75µg/ml (0.2944). Garden Sorrel and Meadow Salsify had the lowest average value both in concentration 50µg/ml: 0.3769 in Garden Sorrel and 0.3256 in Meadow Salsify. The most significant values were detected in Wild Chieve in all concentrations. There were measured following

I: *Content of polyphenols in herbs*

	Ag yoncha	Garden sorrel	Meadow salsify	Wild chive
Gallic acid	24.29	/	**226.17**	29.88
Coumaric acid	/	/	/	30.07
Ferulic acid	/	/	33.19	**131.43**
Rutin	/	/	15.01	3.00
Resveratrol	/	10.09	2.34	/
Vanillic acid	/	34.41	/	/
Sinapic acid	/	**1 507.61**	18.06	/
Catechin	49.73	19.93	/	/
Caffeic acid	30.84	/	46.77	/
Quercetin	**143.00**	/	/	/

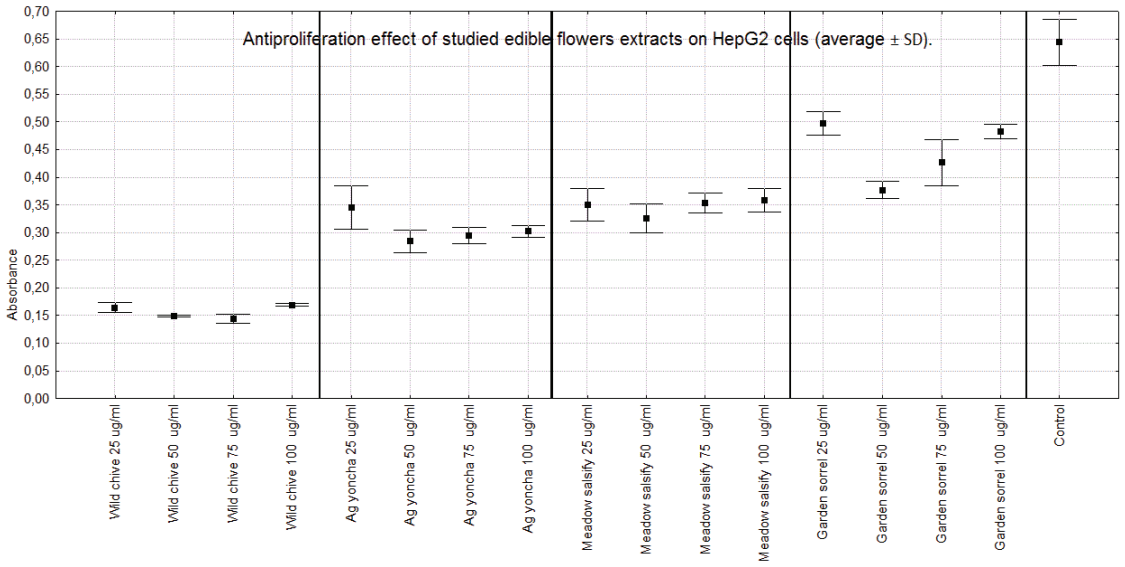
II: *Anti-proliferation effect of edible flowers' polyphenols on HepG2 cells (average ± SD)*

Ag Yoncha 25 µg/ml	0.3453 ± 0.0286**
Ag Yoncha 50 µg/ml	0.2841 ± 0.0556**
Ag Yoncha 75 µg/ml	0.2944 ± 0.0158**
Ag Yoncha 100 µg/ml	0.3023 ± 0.0172**
Garden Sorrel 25 µg/ml	0.4971 ± 0.0328**
Garden Sorrel 50 µg/ml	0.3769 ± 0.0254**
Garden Sorrel 75 µg/ml	0.4268 ± 0.0643**
Garden Sorrel 100 µg/ml	0.4829 ± 0.0659**
Meadow Salsify 25 µg/ml	0.3500 ± 0.0468**
Meadow Salsify 50 µg/ml	0.3256 ± 0.0310**
Meadow Salsify 75 µg/ml	0.3536 ± 0.0273**
Meadow Salsify 100 µg/ml	0.3582 ± 0.0331**
Wild Chive 25 µg/ml	0.1642 ± 0.0139**
Wild Chive 50 µg/ml	0.1492 ± 0.0030**
Wild Chive 75 µg/ml	0.1444 ± 0.0129**
Wild Chive 100 µg/ml	0.1690 ± 0.0034**
Control	0.6440 ± 0.0498*

average values: in concentration 25µg/ml (0.1642), in 50µg/ml (0.1492), in 75µg/ml (0.1444) and in concentration 100µg/ml (0.1690). Nevertheless all of these cases are statistically significant.

DISCUSSION

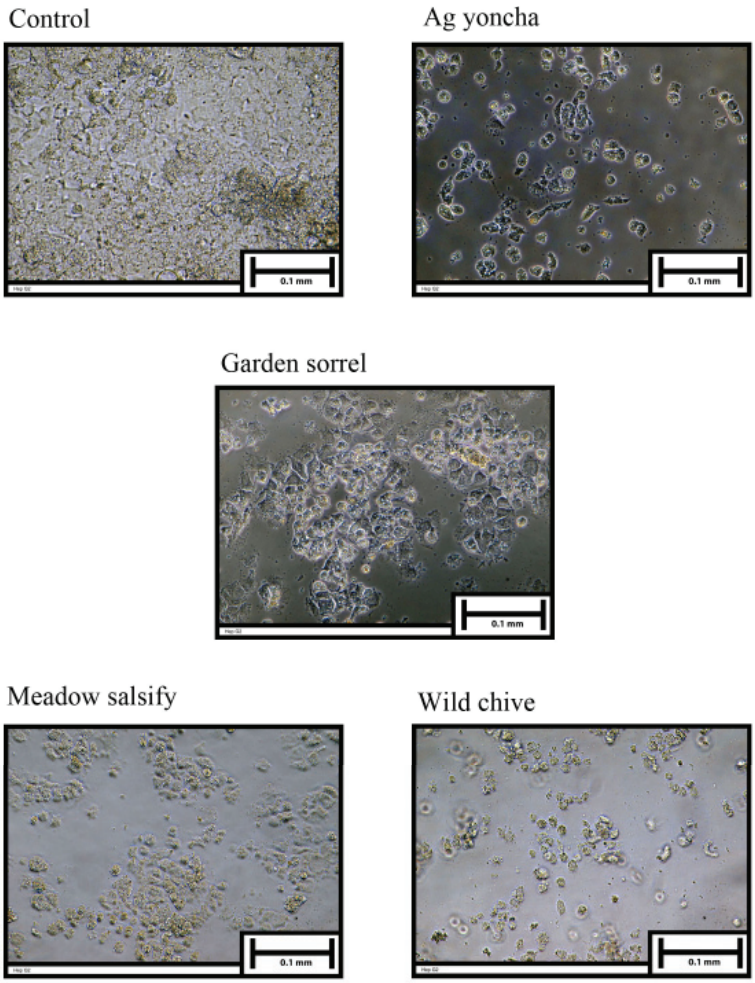
In our study widely used HepG2 cells were used for investigation of anti-proliferation activity associated with polyphenols (Newell *et al.*, 2010; Hai-Bo *et al.*, 2010; Granado-Serrano *et al.*, 2010). Thought, several hundreds of different polyphenols have been identified in plants (Aggarwal and Shishodia, 2006; Scalbert and Williamson, 2000 and Cai *et al.*, 2004) we decided to detect just some of the most important. As can bee seen in Fig. 1, where anti-proliferation activity of extract from Wild chive is presented, the cells incubated in the presence of extract have remarkably lower proliferation compared with control group. Tab. I shows that the highest amount of polyphenols in Wild chive takes ferulic acid which is one of the most common phenolic acids which is present in



1: *Anti-proliferation effect of studied edible flowers extracts on HepG2 cells*

free form in tomatoes, beer, cereal grains (Mateos *et al.*, 2006), orange, carrot, sweet corn and rice bran (Balasubashini, Rukkumani and Menon, 2003). Ferulic acid has a wide range of therapeutic properties against cancer, diabetes, cardiovascular or neurodegenerative diseases (Itagaki *et al.*, 2009). It possesses antioxidant, anti-cancer and anti-inflamatory activities and it has hepatoprotective properties (Hyo-Yeon *et al.*, 2011). Ferulic acid suppressed carcinogenesis in the forestomach, lungs, skin, tongue and colon in experimental animal models (Baskaran *et al.*, 2010). According to Lin *et al.* (2010), ferulic acid has the ability to inhibit cell proliferation and tumour development, which corresponds to our results. Other polyphenols (gallic acid, coumaric acid and rutin) detected in Wild chive have rather low content against ferulic acid.

In another studied edible flower of Ag yoncha, were detected gallic acid, catechin, caffeic acid and quercetin. From which the highest content has quercetin (143.00µg/g). Quercetin is a polyphenolic flavonoid which belongs to the group of phytochemicals (Kim, Kwon, and Jang, 2011). It occurs in fruit and vegetables (Yoshida *et al.*, 1989), buckwheat (Link *et al.*, 2010), red wine, apples, tea (Gosse *et al.*, 2005) and particularly onion (Saunders and Wallace, 2010). It is famous for its anti-carcinogens effects, anti-oxidative activity, inhibition of enzymes that activate carcinogens and interactions with receptors and other proteins (Canivenc-Lavier *et al.*, 1996; Shih, Pickwell, and Quattrochi, 2000; Moon, Wang and Morris, 2006). Most of these effects were investigated *in vitro* or on rodents. Quercetin has chemopreventive effect (Dihal *et al.*, 2006), another studies show its ability to suppress histological tumor marker in the rat colon (Volate *et al.*, 2005; Femia *et al.*, 2003) or in the bone marrow. Other studies describe effects of quercetin in human, for example on lung cancer (De Stefani *et al.*, 1999), chronical diseases as ischemic heart diseases, asthma and diabetes (Knekt *et al.*, 2002) pancreatic cancer in current smokers (Nöthlings *et al.*, 2007) and gastric cancer (Yoshida et al., 1990). Mentioned studies are in agreement with our results. Nevertheless there is a border in the sphere of quercetin activity, because another study shows that quercetin has no positive



2: *Micrographs of HepG2 cell-line cultivated in different extracts (concentration of polyphenols 50µg/ml)*

influence on epithelial ovarian cancer (Murakami, Ashida, and Terao, 2008).

Meadow salsify contained gallic acid, rutin, resveratrol, sinapic acid and caffeic acid for more. The highest amount in Meadow salsify was found to be gallic acid (226.17μg/g), which has significant inhibitory effects on cell proliferation, induced apoptosis in a series of cancer cell lines, and showed selective cytotoxicity against tumour cells with higher sensitivity than normal cells *in vitro* (Salucci *et al*., 2002). Galic acid is presented also by Saxena *et al.* (2008) as a potential compound with anti-cancer activity against hormone-dependent breast cancer, liver and oral cancer cell lines in their study. In the other study Yeh *et al.* (2005) described growth inhibitory effect of phenolic acids including gallic acid on HepG2 cells but at higher concentrations and also they found its increasing enzymatic activity (phenolsulfotransferase), which is important as enzyme in drug metabolism, detoxification and the regulation of intra-tissue active hormone levels. Fig. 1 shows the anti-proliferation activity of extract from Meadow salsify on the HepG2 cells as well.

At last polyphenols contained in Garden sorrel are resveratrol, vanillic acid, sinapic acid and catechin (Tab. I). The highest concentration of polyphenols in Garden sorrel was sinapic acid (483.21μg/g). Fig. 1 shows the Anti-proliferation activity of this edible flower extracts on the HepG2 cells. As can be seen the most significant anti-proliferation activity was found at concentration of 50μg/ml. The morphological differences between HepG2 cells incubated in the presence of edible flowers polyphenols and their comparison with control group are shown in Fig. 2.

It shows differences in morphology between control sample and cells incubated in edible flowers' extracts. Control sample indicate confluent cells while cells incubated in presence of extracts shows decreasing number of cells. Cells do not have their typical profile and their boundaries are less clear.

The anti-proliferative effect of presented polyphenols on HepG2 cells slightly depends on different concentrations. Only in case of Wild chive the effect was independent on applied concentration of polyphenols as similar effect was observed for all concentrations. The anti-proliferative effect of Ag yoncha, Garden sorrel and Meadow salsify was depending on the concentration of polyphenols. This effect may be influenced not only by PF from extracts, but also other active substances which were not determined in this study. The highest inhibition of proliferation of HepG2 cells were in concentration 50μg/ml in all of these cases. Our results show that concentrations of polyphenols higher than 50μg/ml not necessary suppress the proliferation of HepG2 cells. For example, Murugan *et al.* (2010) describe black tea polyphenols at concentration 100μg/ml and their ability to reduce cell viability of HepG2 cells by 60%, which can mean that polyphenols contained in edible flowers used for this study have probably higher anti-proliferative effect. These differences could be caused by different time of incubation or different composition of present polyphenols.

CONCLUSION

Hepatocellular carcinoma belongs to the most common malignant tumor worldwide. This study has demonstrated the influence of herbal polyphenols on the proliferation of HepG2 cells. There is a large scale of studies which were occupied with influece of polyphenols on hepatoma cells but most of them were applied *in vitro* or on rodents. The impact of PF on cancer is not dependent on composition of polyphenols only. There are other factors which can influence it. It is necessary that another researches to discovered more specific effects of polyphenols on human cells will be performed. Chosen edible flowers' extracts have significant anti-proliferation activity, so they can be useful in medical application, for example in cancer prevention and treatment. Other effect of these extracts will be described in the next studies, which will be specialized in lower concentration of PF.

SUMMARY

Cancer is one of the most dangerous diseases in the world. A lot of scientists fight with this disease and try to find methods of its treatment. One of the ways is preventive care about own health. The goal of this study was to determine the anti-proliferative effect of polyphenols, contained in the selected edible flowers (wild chive, meadow salsify, garden sorrel and ag yoncha) on hepatic cells because the hepatocellular carcinoma is the most common malignant tumor worldwide. Polyphenols were extracted from frozen edible flowers by homogenization in methanol, follows extraction and after centrifugation in three repetitions and in the end the extracts were concentrated to 1000μg/ml of polyphenols. The human hepatocellular carcinoma cells were cultivated. The samples of polyphenols were diluted into four concentrations and added to cultivated hepatocellular cells for three days. After this period the anti-proliferative effect were evaluate (by MMT assay). From each edible flower were determined types of polyphenols by chromatography. This concrete found polyphenols and their amount and composition are shown in presented tables. Extract of wild chive has the biggest anti-proliferative effect on hepatocellular cells in comparison with control sample. However, other extract has significant lower proliferation as well. Although, results show that amount and composition of polyphenols, contained in edible flowers, is not important and there are other specific substances in

each flower, which can influence proliferation of cancerous cells, the prominent impact of extracts has been proved. Presented results may be useful in medical application and especially in cancer prevention and treatment, but more studies are necessary.

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Address

Mgr. Dagmar Moravčíková, Institut zdravotnických studií, Ústav porodní asistence, Fakulta humanitních studií, Univerzita Tomáše Bati ve Zlíně, Mostní 5139, 760 01 Zlín, Česká republika, Ing. Zdenka Kuceková, Centrum polymerních materiálů – Fakulta technologická, Centrum polymerních systémů, Univerzita Tomáše Bati ve Zlíně, nám. T. G. Masaryka 275, 762 72 Zlín, Česká republika, Ing. Jiří Mlček, Ph.D., doc. Ing. Otakar Rop, Ph.D., Ústav technologie a mikrobiologie potravin, Fakulta technologická, Univerzita Tomáše Bati ve Zlíně, nám. T. G. Masaryka 5555, 762 72 Zlín, Česká republika, Ing. Humpolíček Petr, Ph.D., Centrum polymerních materiálů – Fakulta technologická, Centrum polymerních systémů, Univerzita Tomáše Bati ve Zlíně. nám. T. G. Masaryka 5555, 760 05 Zlín, Česká republika, e-mail: humpolicek@uni.utb.cz