Antioxidant and genoprotective properties of extracts from edible flowers

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Summary
Enrichment of diet with edible flowers as sources of antioxidants may have beneficial effect on human health. The goal of the study was to check whether the popular edible flowers can provide protection against oxidative DNA damage, which is the main risk factor of aging and initiation of carcinogenesis. The analysis included hydrolates, 40% tinctures and essential oils from edible flowers *Alcea rosea, Bellis perenis, Calendula officinalis, Calluna vulgaris, Centaurea cyanus, Citrus aurantium, Heliantus annuus, Hibiscus sabdarifa, Jasminum grandiflorum, Lavandula augustifolia, Matricaria chamomilla, Primula vulgaris, Rosa centifolia, Rosa damascena, Rosa rugosa, Sambucus nigra, Tagetes patula, Trifolium pratense* and *Viola cornuta*. Antioxidant power, polyphenol content, cytotoxicity and DNA damage level were analysed. Oxidative DNA damage was induced by hydrogen peroxide. Most of the flowers acted as chemopreventive agents. Roses, French marigold, lavender, heather, elderflower, horned pansy and cornflower can be particularly useful in protecting human DNA against oxidative damage. The best antioxidant and genoprotective properties were observed in *Rosa* genus.

Keywords
edible flowers; *Rosa*; antioxidant; reduction power; DNA damage; comet assay, chemoprevention

Edible flowers are becoming more and more popular food ingredients in European cuisine. The flowers are used as the garnish, seasoning or a basic component of dish. Some of them are present in European cuisine for ages like lavender or roses. Moreover, many of these flowers have cosmetic or phytotherapeutical use with long tradition in Europe, Asia and North America. Essential oils from flowers are used for ages in perfumery and aromatherapy. Lavender, cornflower, orange blossom, jasminum and rosal hydrolates are used in cosmetics due to their soothing and calming effects [1, 2]. Pot marigolds, daisies and chamomile are used for their anti-inflammatory properties. Red clover is known for its estrogenic action. All those flowers used in traditional medicine, cosmetics and phytotherapy became, in last decade, very common as new diet ingredients. As dietary species richness and biodiversity correlate with nutritional value of food [3, 4], new diet components are very welcome but the flowers arouse interest in their possible health benefits and usage in chemoprevention as there is an increasing demand for natural food antioxidants [5–8].

Flowers are the reproductive structures of angiosperm plants. Petals forming corolla do not take part in the reproduction process directly but they are responsible for attracting pollinating animals or mostly insects. Flower petals are usually rich in colorants and fragrances. Yellow and orange blossoms like dandelions, pot marigold, French marigolds are rich in carotenoids, in particular carotens (α-, β-, γ-, δ-, ε- and ζ-carotene) and xanthophylls (lutein, zeaxanthin, neoxanthin, violaxanthin, flavoxanthin, α- and β-cryptoxanthin). Red, white, purple or blue petals are rich in different types of anthocyanins. Most frequently occur the glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. Both carotenoids and anthocyanins are strong antioxi-

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dants [9]. These compounds are known for their anti-inflammatory, anticancer and antiproliferative properties and, moreover, polyphenols participate in activation of phase II xenobiotics metabolizing enzymes [10]. These compounds also inhibit cancer cell proliferation and transformation, tumour invasion and angiogenesis [11, 12] as well as exhibit protective properties against vascular disease [13] and are useful agents against obesity [14, 15].

Essential oils are hydrophobic aromatic compounds from plants. Chemically, they are mixtures of monoterpenes, terpene esters, monoterpenols, sesquiterpenes, terpenoid oxides and others compounds like ketons and phenethyl alcohol. Essential oils are produced by distillation, expression or solvent extraction. The hydrolates (hydrosols) are side products of essential oils distillation. The active compounds of essential oils like geraniol, citrenollol, nerol or phenethyl alcohol are known for their antibacterial [16], antiviral and therapeutic or preventive effects on different types of cancer, including breast, lung, colon, prostate, pancreatic and hepatic cancer [17].

The aim of the study was to analyse whether edible flowers may give a hint of protection against oxidative stress and DNA damage, which are important factors of aging and initiation of carcinogenesis. As different types of extracts differ due to the contents of active phytochemicals, we analysed hydrolates, alcohol extracts and essential oils from edible flowers popular in European cuisine.

MATERIALS AND METHODS

Flower extracts

Hydrolates from cornflower (*Centaurea cyanus*), bitter orange blossom (*Citrus aurantium*), jasminum (*Jasminum grandiflorum*), lavender (*Lavandula augustifolia*), chamomile (*Matricaria chamomilla*), cabbage rose (*Rosa centifolia*) and damask rose (*Rosa damascena*) were obtained from Zrób Sobie Krem (Prochowice, Poland) and Ecospa (Warszawa, Poland).

Esential oils from bitter orange blossom (*Citrus aurantium*, Neroli oil), jasminum, lavender, cabbage rose and damask rose were obtained from Zrób Sobie Krem and Ecospa. The essentials oil were dilluted in ethanol to 1% and 0.1% concentrations.

Tinctures, i.e. 1% alcohol extracts, were prepared by maceration of 1 g of dried flower petals in 100 ml of 40% ethanol for 14 days. Damask rose dried petals were obtained from Bulgaria (Ecospa), jasminum dried petals from Marocco (Ecospa). The other flower petals, namely, black flowers of hollyhock (*Alcea rosea*), daisy (*Bellis perenis*), pot marigold (*Calendula officinalis*), heather (*Calluna vulgaris*), cornflower, sunflower (*Heliantus annuus*), sorrel (*Hibiscus sabdarifa*), lavender, chamomile, primrose (*Primula vulgaris*), cabage rose, beach rose (*Rosa rugosa*), elderflower (*Sambucus nigra*), French marigold (*Tagetes patula*), red clover (*Trifolium pratense*) and blue flowers of horned pansy (*Viola cornuta*) were gathered in Lesser Poland Voivodship (Małopolska) in suburban regions and air dried.

All additional chemicals were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

Ferric reducing antioxidant power

The ferric reducing antioxidant power (*FRAP*) as the ability to reduce Fe^{3+} ions to Fe^{2+} was determined according to Benzie and Strain [18] with minor modifications. Briefly, the calibration curve was plotted using standard $FeSO_4$ solution. $FeSO_4$ solutions (0–1 mmol·l^{-1}) were mixed with FRAP 1 solution (300 mmol·l^{-1} acetate buffer, 10 mmol·l^{-1} 2,4,6-tripyridyl-*s*-triazine, TPTZ) and incubated for 20 min at room temperature in the dark. FRAP 2 solution (300 mmol·l^{-1} acetate buffer, 10 mmol·l^{-1} TPTZ, 20 mmol·l^{-1} $FeCl_3$) was added to the 1% extracts. Afterward, the mixture was allowed to stand for 20 min and absorbance was measured at 593 nm using Spectra Fluor Plus instrument (Tecan, Männedorf, Switzerland) using Magellan software (Tecan). The ascorbic acid water solution in concentration 1 mg·ml^{-1} was used as an antioxidant standard and the results were expressed as the percentage of ascorbic acid antioxidant power. The samples were analysed in triplicate.

Total phenolic content – Folin-Ciocalteu's assay

Total phenolic group content in flower extracts was measured by the standard Folin-Ciocalteu method according to Singleton and Rossi [19]. Briefly, 100 μl of aliquots of standard quercetin solutions (0–0.5 mmol·l^{-1}) and/or tested extracts (1%) were placed into the test tubes and mixed with 750 μl of Folin Ciocalteu's reagent. After 5 min, 750 μl sodium carbonate (60 g·l^{-1}) was added. The incubation was performed for 90 min at room temperature in the dark. Intense blue colour was developed. After incubation, absorbance was measured at 750 nm in spectrophotometer Rayleigh UV1800 (Rayleigh Instruments, Chelmsford, United Kingdom). The samples were measured in triplicates. The blank sample was measured using reagents with solvent. The calibration curve was plotted using standard quercetin

solutions. The data of total polyphenol contents of extracts were expressed as milligrams of quercetin equivalent per millilitre of sample.

Cell isolation and treatment

Human peripheral blood from healthy volunteers was obtained from Regional Center of Blood Donation and Treatment in Krakow, Poland. Peripheral blood lymphocytes were isolated using the gradient centrifugation method on Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's instructions and were frozen at –80 °C in 50% fetal bovine serum (FBS), 40% Roswell Park Memorial Institute 1640 medium (RPMI) and 10% dimethylsulfoxide (DMSO). Before each experiment, lymphocytes were thawed in RPMI medium with 50% FBS and centrifuged at 135 ×*g*, at 4 °C, for 5 min. Subsequently, cells were seeded into 96-well plates at a density of 1×10^4 cells per well in RPMI with 10% FBS and incubated in 37 °C, 5% CO_2 for 30 min. Next, cells were incubated for 1 h in 100-fold diluted (in cell-culture medium) flower tinctures or hydrolates, and 1000-fold diluted essential oils for 1 h and for 24 h at 37 °C in 5% CO_2.

Cell viability – double-staining fluorescence assay

The cytotoxicity of extracts was evaluated using the fluorescein diacetate (FDA) and ethidium bromide (EtBr) differential staining. Cells were seeded at a density of 2×10^4 cells per well in 96-well plates for 1 h and 24 h, and 1% extracts were added to the respective wells. Staining probes was prepared by mixing 5 mg·ml^{-1} FDA in acetone, 200 μg·ml^{-1} EtBr in phosphate-buffered saline (PBS) and PBS without Ca^{2+} and Mg^{2+}. Samples were mixed with FDA/EtBr and placed on microscope slides. The green labelled cells are considered as metabolically active and the red labelled cells are taken as dead. At least 100 randomly selected cells are analysed per slide, using fluorescence microscope IX50 (Olympus, Tokio, Japan) and the procedure was repeated in three independent experiments.

DNA damage level – comet assay

The single cell gel electrophoresis (comet assay) procedure was performed to examine the amount of endogenous oxidative DNA damages and single-stranded breaks in lymphocytes according to Kapiszewska et al. [20]. Pattern of DNA migration through the electrophoresis gel in fluorescence microscope resembles a comet with a head formed by undamaged DNA and a tail of migrating fragments of damaged DNA. The fluorescence intensity of the comet tail depends on the amount of DNA damages in nucleus. Lymphocytes were placed in a 96-well plate at a density of 2×10^4 cells per well and were treated with 1% flower extracts solution for 1 h. Oxidative DNA damage was induced by cell incubation in 25 μmol·l^{-1} hydrogen peroxide for 5 min. After incubation, cells were centrifuged (135 ×*g*, 5 min, 4 °C) and suspended in PBS. Samples were placed in a water bath (37 °C) and mixed with low melting point agarose (LMPA) solution. Suspension from each sample was placed on normal melting point agarose (NMPA) coated slides, covered with coverslips and immediately transferred on ice. After the coverslips removal, slides were placed in lysing buffer (2.5 mol·l^{-1} NaCl, 100 mmol·l^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol·l^{-1} tris(hydroxymethyl)aminomethane (Tris), 10% DMSO and 1% polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X100); pH 10) at 4 °C for 1 h. Then, the slides were washed 3 times in 0.4 mol·l^{-1} Tris-HCl (pH 7.4) and placed in electrophoresis tank filled with fresh cold electrophoresis buffer (300 mmol·l^{-1} NaOH, 1 mmol·l^{-1} EDTA). The slides were kept in the alkaline buffer for 40 min to allow DNA strands relaxation. Subsequently, electrophoresis was conducted for 30 min (0.74 V·cm^{-1}, 300 mA) and then the slides were washed with 0.4 mol·l^{-1} Tris-HCl (pH 7.4).

The results from two independent experiments were expressed as tail DNA content (*TDC*, percentage of DNA fluorescence in the tail of total fluorescence of DNA in the comet) after propidium iodide staining in fluorescence microscope IX50 equipped with an excitation filter of 515–560 nm with a barrier filter of 590 nm at magnification of 200×. The images were analysed by Comet Plus 6 software (Theta Electronics, Gröbenzell, Germany) and *TDC* was automatically calculated for random 100 comets from each sample.

Statistical analysis

Basic statistical analysis was performed using Statistica 9.0 (StatSoft, Palo Alto, California, USA). For parameters comparison, ANOVA test was used under the condition of positive homogenity of variance in Levene's test. The posteriori Tukey's test was performed. A p value of 0.05 was considered the cut-off for significance. The correlation analysis was performed with linear regression and the force of correlation was established by Pearson's correlation factors.

RESULTS

Antioxidant power and polyphenol content

The results obtained by FRAP method revealed that hydrolates had the lowest antioxidant power among the analysed extracts (Fig. 1). Among the hydrolates, the strongest antioxidant abilities had the damask rose hydrolate. The essential oils from cabbage rose and damask rose revealed the strongest antioxidant abilities among all extracts. However, not all essential oils acted as antioxidants, as jasmine and lavender oils were found to be very weak antioxidants. Also, the tincture from jasmine flowers did not exhibit antioxidant activity. Tinctures of black hollyhock, hibiscus, chamomile, sunflower, primrose and daisy had rather weak abilities, equivalent to less than 50% of 1 mg·ml^{-1} ascorbic acid solution. Tinctures from cornflower, lavender, blue horned pansy, heather, elderflower, French marigold, red clover and pot marigold revealed strong antioxidant abilities, with the highest values being determined for beach rose and damask rose tinctures.

Results on the correlation between antioxidant power (determined by FRAP method expressed in percent of 1 mg·ml^{-1} ascorbic acid solution) and total phenolic content (determined using Folin-Ciocalteu method) of flower extracts, for hydrolates, essential oils and tinctures (p = 0.00007), are shown in Fig. 2. The *Rosa* genus extracts were characterized not only by the strongest antioxidant activities but also by the highest concentrations of polyphenols. The *Rosa* extracts were represented by four outstanding points at the right side of Fig. 2. Moreover after exclusion of rosal extracts, the correlation was even stronger (p = 0.00000; R^2 = 0.6719; y = 5.4025 + 0.7236x).

Impact on cell viability

Vitality of cells after treatment for 1 h and after 24 h with 1% flower tinctures diluted 100-fold in the medium revealed that they did not affect cell vitality. The mean cell vitality after 1 h incubation in flower tinctures was (93.9 ± 2.6) % and, after 24 h, (73.0 ± 19.5) %, 100% being the vitality of control cells incubated in RPMI with 10% FBS. The 1% dilution of essential oil was lethal for lymphocytes after 1 h incubation, and 0.1% was lethal after the incubation time of 24 h. The results are not visualised according to their homogenity.

Because of cytotoxicity, essential oils were excluded from further investigations regarding their influence on DNA. The hydrolates were also excluded due to their weak antioxidant activity and according to the fact that they were not standardized during preparation.

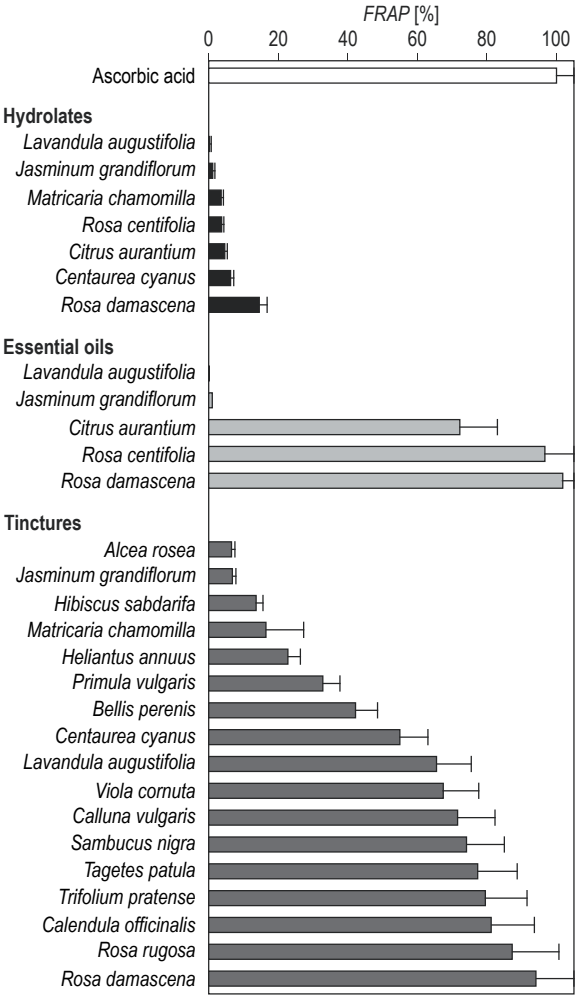

Fig. 1. Antioxidant power of flower extracts.

FRAP – ferric reducing antioxidant power of sample expressed as percent of antioxidant abilities of ascorbic acid solution (1 mg·ml^{-1}).

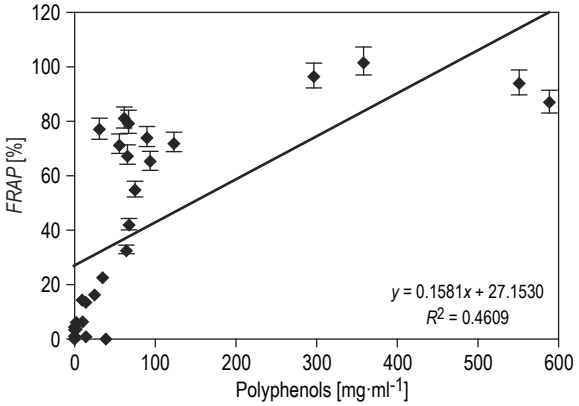

Fig. 2. Correlation between antioxidant power and concentration of polyphenols in flower extracts.

FRAP – ferric reducing antioxidant power of sample expressed as percent of antioxidant abilities of ascorbic acid solution (1 mg·ml^{-1}).
Concentration of polyphenols is expressed as milligrams of quercetin equivalents per millilitre of sample.

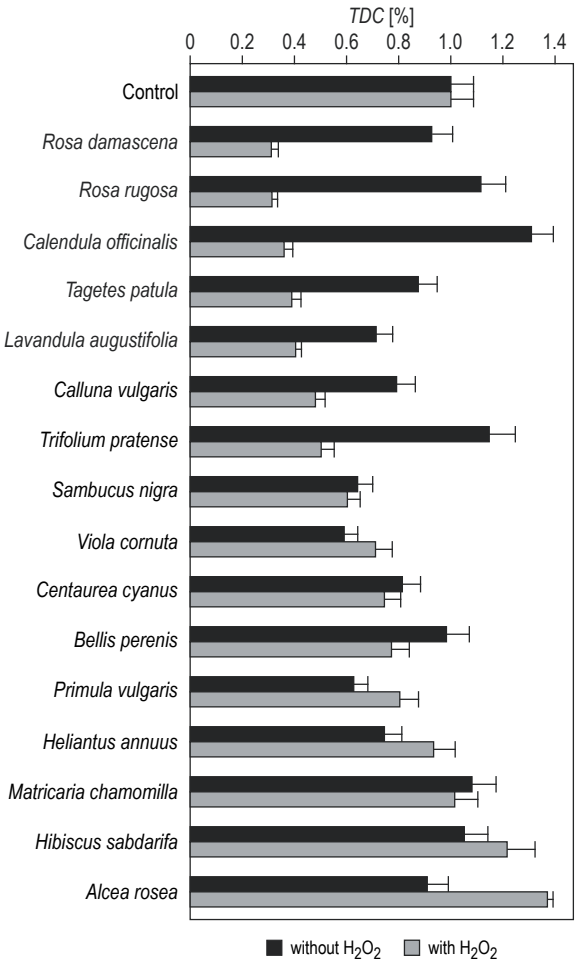

Fig. 3. DNA damage in lymphocytes treated with flower extracts.

TDC – tail DNA content, the level of DNA damage measured by comet assay expressed as percentage of DNA fluorescence in comet tail to total fluorescence of the comet.

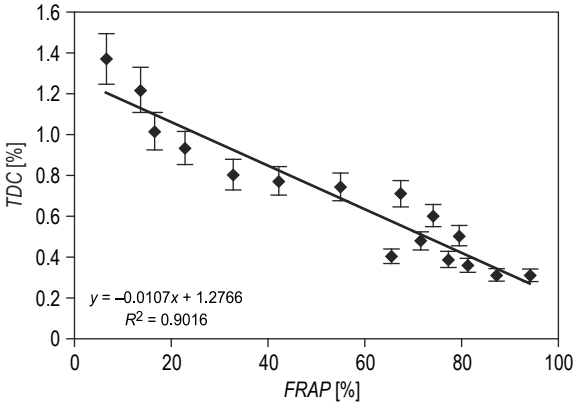

Fig. 4. Relation of genoprotective abilities and antioxidant power of flower extracts.

TDC – tail DNA content, the level of DNA damage measured by comet assay expressed as percent of DNA fluorescence in comet tail to total fluorescence of the comet.
FRAP – ferric reducing antioxidant power expressed as percent of antioxidant abilities of ascorbic acid solution (1 mg·ml^{-1}).

DNA damage

The comet assay revealed that the flower extracts did not induce DNA damage in lymphocytes (1% flower tinctures diluted 100-times in the medium), which is presented in Fig. 3. The results of DNA damage were expressed as *TDC* of treated cells in reference to untreated cells. The flower extracts in Fig. 3 were ordered according to the decreasing protective abilities against induced oxidative DNA damage. The exception were the tinctures from pot marigold and red clover, which seemed to induce DNA damage in lymphocytes untreated with H_2O_2 ($p = 0.003$ and $p = 0.018$, respectively). However, they effectively protected DNA of lymphocytes from damage induced by 25 μmol·l^{-1} H_2O_2 ($p = 0.0001$ and $p = 0.0004$, respectively).

The highest protective effect of flower extracts against non-induced DNA damage was observed for horned pansy ($p = 0.0011$), primrose ($p = 0.008$), elderflower ($p = 0.002$), lavender ($p = 0.0018$), French marigold ($p = 0.027$) and heather ($p = 0.034$). Moreover, flower petal extracts from damask rose ($p = 0.0001$), beach rose ($p = 0.0001$), pot marigold ($p = 0.0001$), French marigold ($p = 0.0031$), lavender ($p = 0.0049$), heather($p = 0.0073$), red clover ($p = 0.018$), elderflower ($p = 0.004$), blue horned pansy ($p = 0.016$), cornflower ($p = 0.032$) and daisy ($p = 0.042$) significantly protected DNA of lymphocytes from oxidative damage induced by hydrogen peroxide. However, roselle ($p = 0.034$) and black hollyhock ($p = 0.012$) extracts enhanced pro-oxidative action of hydrogen H_2O_2 and induced increased oxidative DNA damage in lymphocytes.

There was no correlation of the level of oxidative damage in DNA of lymphocytes exposed to 1% flower extracts diluted 100-fold depending on polyphenol concentration in extracts ($p = 0.231$; $R^2 = 0.128$). No such correlation was observed also regarding FRAP ($p = 0.740$; $R^2 = 0.008$). However, there was a strong negative correlation between the level of DNA damage (expressed as *TDC*) induced with 25 μmol·l^{-1} H_2O_2 ($p = 0.0004$; $R^2 = 0.697$; $y = 1.2957 – 0.0096x$) and polyphenol concentration (after exclusion of outstanding results for *Rosa*) as well as with FRAP ($p = 0.00000$; no exclusions), which is shown in Fig. 4.

DISCUSSION

The obtained results proved that flower extracts have antioxidant capacities. The antioxidant potential differed among species and extract types. The weakest antioxidant power was re-

vealed in hydrolates and the strongest in essential oils. The aqueous-alcoholic extracts prepared from rose petals were characterized by a strong antioxidant activity. If we consider that the composition of extracts depends on the method of extraction, these results are not surprizing [21–23]. However, essential oils diluted 100- and 1 000-fold were cytotoxic to lymphocytes.

Decision of choosing 40% tinctures for experiments with cells was justified by the fact that ethanol-aqueous extract would be the most comprehensive form of extract that contains both hydrophilic and hydrophobic constituents. As it was assumed, the edible flowers are usually consumed as a part of a complex dish, therefore they are eaten accompanied by fats and water, thus polar and non-polar types of constituents are both possible to be ingested.

The most interesting results were obtained for different extracts from *Rosa* genus. *Rosa rugosa* was confirmed as a strong antioxidant [5, 7, 24–27]. As it was shown, lavender oil showed a strong antibacterial activity whereas hydrosol did not [28]. In our study, neither essential oil neither hydrolates did show a good antioxidant activity, but the lavender tincture was a good antioxidant. This confirms that the extraction method is crucial for the extract composition. Furthermore, the results suggest that antioxidant constituents of lavender may by thermolabile.

Jasmine is considered a source of antiseptic, antiviral and anti-inflammatory components like oleuropein [29]. However, our results did not reveal antioxidant properties of jasmine complex extracts. The obtained results confirmed that cornflower possess rather weak antioxidant capacity and protective abilities, as well as a low content of phenolics [1, 7]. A similar situation was in case of common daisy and primrose extracts, which were characterized by moderate antioxidant and genoprotective abilities. Blue-flowered horned pansy was shown to be a strong antioxidant and a good source of polyphenols. As it is a close relative to garden pansy (*Viola*×*wittrockiana*) and Johnny-jumps-up (*Viola tricolor*), it is justifiable to compare their results [30, 31] and to corroborate the good antioxidant capacities of *Viola* family. French marigold was found to be a good source of antioxidants with a high content of flavonoles [32]. Pot marigold has previously shown cytotoxic effects on mammalian cell lines [33]. However, our results revealed this effect also on non-transformed human lymphocytes. Nevertheless, pot marigolds exhibit antioxidative properties as it was presented by other researchers [7, 33, 34]. Sunflower petals, holy hock, chamomile and hibiscus did not show any antioxidant activity and these flower extracts did not protect DNA against oxidative damage. This is in contradiction with other studies [35, 36].

Obviously, not only polyphenols have impact on antioxidative properties of flowers. Yellow and orange blossoms contain carotenoids, which may affect the relation between antioxidant properties of flower extracts and the polyphenol content. In our study, the good example for this case could be the results obtained for French marigold in which FRAP was definitely strongly outstanding from correlation between antioxidant power and polyphenol content suggesting that the high concentration of carotenoids, probably lutein, could be responsible for this observation [37].

The case of heather, pot marigold and lavender suggested that not only the direct antioxidant activity is responsible for cell protection, but the possible mechanisms are also changes in the gene expression or changes in the activity of enzymes [38, 39].

Protective abilities of flower extracts against DNA oxidative damages were primary related to their antioxidative properties as it was proven by strong negative correlation between those parameters.

As it was shown for the effective prevention for cancer, one of the most important factors is that the food is rich in antioxidants but not in dietary supplements [40]. One of the reasons is the variety of chemopreventive agents that can be found in food and that such diversity is not offered by supplements [41]. In order to improve antioxidant properties of meals [42], addition of edible flowers may give some supplementary chemoprotection to human cells. Studies in South America revealed that consumers were ready to buy food with edible flowers due to their health benefits [43]. Therefore, this is a feature that should be accented in promotion of flowers as food. It is also worth emphasizing that, besides the strong antioxidant and chemopreventive properties, these flowers taste good.

CONCLUSION

Our results show that flowers of damask rose, beach rose, French marigold, lavender, heather, elderflower, horned pansy and cornflower can be particularly useful in protecting human DNA against oxidative damage. Further research with animals is suggested to study the absorption and the action of flower extracts in vivo.

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