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Edible Flowers Extracts as a Source of Bioactive Compounds with Antioxidant Properties—In Vitro Studies

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Featured Application: ***Trifolium repens*** **and** ***Trifolium pratense*** **extract can be seen as a source of antioxidant com-pounds even after heating (extraction) for human diet or as functional food in food industry.**

Abstract: Edible plants began to play an important role in past decade as a part of therapy, a recovery process or a healthy life style. The availability and relatively low price of the raw material, as well as proven bioactive health benefits, are key to consumers' choice of nutrients. The red clover (*Trifolium pratense*) is a popular plant with healthy properties such as antiseptic and analgesic effects. The less known white clover (*Trifolium repens*), a fodder and honey plant, has anti-rheumatic and anti-diabetic properties. Both species may serve as a potential source of bioactive substances with antioxidant properties as a food additive or supplement. The study material consisted of flower extracts of *Trifolium repens* and *Trifolium pratense*. The total content of polyphenols and DPPH (2.2-diphenyl-1-picrylhydrazyl) and ferric reducing antioxidant power (FRAP) were measured using spectrophotometry methods. Oxidative stress in THP1 cells was induced via sodium fluoride. Subsequently, flower extracts were added and their influences on proliferation, antioxidant potential and the activity of antioxidant enzymes were evaluated. The extracts have a high total content of polyphenols as well as high antioxidant potential. We also demonstrated positive extracts impact on cells proliferation, high antioxidant potential and increasing activity of antioxidant enzymes on cell cultures under high oxidative stress induced by fluoride. Both red clover and the less known white clover may serve as valuable sources of antioxidants in the everyday diet.

Keywords: natural compounds; antioxidant; oxidative stress; red clover; white clover; fluoride; polyphenols

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1. Introduction

The increased generation of reactive oxygen species (ROS) may be caused by multiple factors, such as unhealthy lifestyles (alcohol, nicotine, stress) as well as food and environmental contamination. Excessive release of ROS may cause severe oxidative damage, leading to numerous disorders [1]. Oxidative and inflammatory processes are the basis for the pathogenesis of numerous civilisation diseases, including atherosclerosis, diabetes, neurodegenerative illnesses and even obesity [2–4]. The solution to maintaining the appropriate oxidative balance of the organism is a high supply of antioxidants, antioxidants from external sources, with the aim of equalizing and preventing oxidative processes. It is also important to maintain a correct lifestyle, free from stress-inducing factors [5]. Plant resources are an easily accessible natural antioxidant. In recent years, edible plants have been increasing in popularity due to their rich chemical content and healthy properties [6–10].

The clover (*Trifolium* L.) belongs to the *Fabaceae* family and includes about 300 species present in Europe, Asia and North America [11]. Two species of these perennial plants are common in Europe: the red clover (*Trifolium pratense*) and the white clover (*Trifolium repens*). They grow in the wild, but are also farmed and used mostly as fodder and honey plants. They are also used in phytotherapy and in the kitchen—as an ingredient to various meals and drinks, not only due to their aesthetic value, but also nutritional properties [11]. The white clover is mainly used as a honey and fodder plant. It is valued for its high protein content. In traditional medicine, it was an ingredient of herbal blends used for anti-rheumatic, anti-diarrheal and anti-arthritic purposes [11]. Red clover, the type that is mainly used in medicine, is recommended for numerous ailments, including sore throat, pneumonia, meningitis, pulmonary diseases and skin problems, disorders of the reproductive system, diabetes and even obesity. Numerous studies have proved that the plant facilitates coughing, has antiseptic and anaesthetic properties, and that it also reduces the level of total cholesterol [11–17].

Despite studies concerning the antioxidant and anti-inflammatory properties of the red clover, there are still insufficient articles on the influence of this plant on macrophages in the model of induced oxidative stress. ROS may contribute to the activation of numerous mechanisms contributing to the pathogenesis of atherosclerosis, such as: the dysfunction of endothelial cells, monocyte recruitment and their differentiation into macrophages, as well as the stimulation of inflammatory response. Moreover, there are very little data confirming the beneficial influence of the other, less known, but easily available species—the white clover. Therefore, the aim of the study was to determine the effect of white clover and red clover ethanolic flower extracts, rich in polyphenols, on antioxidant potential and protection effect in THP-1. THP 1 cells, monocytes, after transformation into macrophages are involved in reactions related to oxidative stress and inflammation, and also are implicated in many civilization diseases including atherosclerosis.

2. Materials and Methods

2.1. Plant Material

The flowers of white clover (*Trifolium repens)* and red clover (*Trifolium pratense*) gathered at the blooming period from the green areas (Poland) served as the study material for this research. The collected flowers underwent lyophilisation in a lyophilisator (0.735 mmHg/−20 °C; Alpha 1-2 LD plus), and then they were subjected to homogenisation by grinding in a food homogenizer to the form of powder (FOSS 2094).

2.2. Preparation of Alcoholic Extract

Extraction was carried out in the following manner: 1.5 g of ground plant material was placed in a thimble, which was then transferred to a Soxhlet apparatus. The Soxhlet apparatus was placed between a 250.0 cm^3 round bottom flask containing 150.0 mL of ethanol (96%) and a reflux condenser. The contents of the round bottom flask were boiled and extracted for 5 h. Then, the mixture was cooled down to room temperature and filtered. Then alcohol was evaporated. The obtained alcohol extract was placed in a plastic vial and then stored at −20 °C until used. Next before analysis extracts were dissolved in 96% ethanol (for spectrophotometric analysis) and in dimethyl sulfoxide (DMSO, for cell culture) at the 100.0 mg/L concentration.

2.3. Spectrophotometric Analysis of Ethanol Extracts Prepared from the Flowers of White Clover and Red Clover

2.3.1. Antioxidant Activity of Extracts by the DPPH Methods

The antioxidant activity of samples was measured with spectrophotometric method using synthetic radical DPPH (2.2- diphenyl-1-picrylhydrazyl) from Sigma according to Brand-Williams et al. and Pekkarinen et al. [18,19]. 96% ethanol, 1 mL of 0.3 mM solution of DPPH in 96% ethanol, and 0.1 mL of the test sample were introduced into the vial in *v/v* ratio 29:10:1. The prepared solution after mixing was placed for 30 min in a dark

place. During this time, the so-called A0 solution was prepared by mixing 96% ethanol and 0.3 mM solution of DPPH in *v/v* ratio 3:1. 96% ethanol was used as a reference solution. Before the measurement, the vial contents were thoroughly mixed, poured into cuvettes. The spectral absorbance was immediately measured at 518 nm. All tests were performed in triplicate. Antioxidant potential of tested solutions has been expressed by the percent of DPPH inhibition, using the formula:

$$\% \text{ inhibition } = \frac{A0 - As}{A0} \quad (1)$$

where:

A0 absorbance of DPPH solution at 518 nm without tested sample
As absorbance of DPPH solution at 518 nm with tested sample

2.3.2. Determination of Reduction Potential of Extracts by the Ferric Reducing Antioxidant Power (FRAP) Method

The ferric reducing antioxidant power (FRAP) method is used to determine the total reduction potential, based on the ability of the test sample to reduce Fe^{3+} ions to Fe^{2+} ions. The FRAP unit determines the ability to reduce 1 mole Fe^{3+} to Fe^{2+} according to Benzie and Strain [20,21]; 3 mL of the FRAP reagent, consisting of tripyridyltriazine (TPTZ) solution, acetate buffer once $FeCl_3$, the test sample and distilled water were introduced into the vial. The prepared solution after mixing was placed for 5 min at 37 °C.

Absorbance at 593 nm was measured (Agilent 8453UV). All tests were performed in triplicate.

2.3.3. Determination of the Total Polyphenols Content (TPC) in Extracts

Determination of polyphenols was performed according to ISO 14502-1 and Singleton, Rossi method using Folin–Ciocalteu reagent [22]; 5.0 mL of a Folin–Ciocalteu solution (10%) and 1.0 mL of test sample were introduced into the vial. The vial contents were thoroughly mixed and after 5 min. 4.0 mL of 7.5% Na_2CO_3 solution was added and incubated for 60 min. at room temperature. Reference solution was prepared the same way but instead of tested sample distilled water was added. Absorbance at 765 nm was measured (Agilent 8453UV). All tests were performed in triplicate. The results are shown in mg/L gallic acid (GAE).

2.4. Cell Cultures

The monocytic cells, THP-1 line (ATCC, USA), cultivated on 6-well plates (2×10^6 cells) were differentiated into macrophages by adding 10.0 µl of phorbol ester (PMA, 100nM) (Sigma-Aldrich) to the culture medium RPMI (Wytwórnia Szczepionek i Surowic, Poland) that included 10% of the fetal bovine serum (FBS) (Gibco, Loughborough, UK), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Sigma-Aldrich, Poland). The cells were incubated in standard conditions (37 °C and 5% CO_2) for 24 h. Next, the adherent macrophages were washed three times with buffered saline solution without calcium and magnesium (phosphate-buffered saline, PBS) (Wytwórnia Szczepionek i Surowic BIOMED, Warszawa, Poland). The cells were incubated for 48 h with the factors: NaF at a concentration of 10 µM, causing oxidative stress [23,24] and extracts of clover (red and white) with a final concentration of 100 mg/L. Flower extracts were dissolved in DMSO (Chempur) at a concentration that after addition to the cell culture not exceeding 1% [25]. The positive control for fluoride was distilled water, for the extracts-(DMSO), while for both variants introduced to the culture it was simultaneously water and DMSO. The concentrations of the added fluorides were chosen on the basis of the results of the analyses of human serum carried out by other author [26]. After 48 h of incubation, the cells were scrapped and centrifuged with PBS solution, and the resulting cell lysates were used in spectrophotometric measurements. Protein concentration was measured by using a Micro BCA Protein Kit (Thermo Scientific Pierce Biotechnology, Waltham, MA, USA).

2.5. Measurement of the Total Antioxidant Capacity and the Activity of Antioxidant Enzymes

The antioxidant potential was measured using the Antioxidant Assay Kit (Cayman Chemical Company, MI, USA). The kit measures the total antioxidant capacity of cell lysates. The activity of antioxidant enzymes was measured by using of the Superoxide Dismutase Assay Kit, Catalase Assay Kit (Cayman Chemical Company). The antioxidant potential was measured spectrophotometrically at 405 nm and presented in the form of mM. Catalase (CAT) activity was measured spectrophotometrically at 540 nm and is presented in nmol/min/mL/protein. The activity of superoxide dismutase (SOD) was measured by spectrophotometry by estimating the amount of superoxide anion in room temperature at 440 nm and presented in [U/mL]. The intensity of the reaction produced in the reaction was measured in a microplate reader (Biogent, Józefów, Poland).

2.6. Determination of Cell Proliferation

Proliferation of macrophages was measured using the BrdU test, based on determining the influence of the studied factors on the synthesis of DNA in cells by measuring the degree of bromodezoxyuridine (BrdU) binding. THP-1 cells with a density of 1×10^5 cells/well were poured into 96-well plates. The next day, the culture fluid was collected and the cells were treated with test substances (extracts with a concentration of 100 mg/L and NaF 10 uM) suspended in fresh culture medium. After 12 h, the BrdU test was performed according to the manufacturer's instructions (BrdU Cell Proliferation Assay Kit, MyBioSource). The intensity of the color was measured in a microplate reader (Biogent, Józefów, Poland) at 450 nm.

2.7. Statistical Analysis

The statistical analysis was performed using Statistica Stat Soft 13.5 and Microsoft Excel 2017. The results are expressed as mean values and standard deviation (SD). Three samples were analysed and all the assays were carried out at least in duplicate. To assess the differences between examined parameters, the Tukey post-hoc test was used. Differences were considered significant at p value ≤ 0.05. To control type I errors, the false discovery rate (FDR) approach was used. The calculations were performed using the p. adjust function of the stats package in R Foundation for Statistical Computing, Vienna, Austria, https://cran.r-project.org (accessed on 24 February 2021).

3. Results

3.1. Analysis of the Antioxidant Properties of Flower Extracts

The antioxidant potential measured by DPPH, expressed as a percentage of DPPH radical inhibition, was 4.48 $\pm$ 0.21% for red clover flower extract, and 3.86 $\pm$ 0.16% for white clover flower extract. The reductive potential measured through FRAP was 100.20 $\pm$ 4.96 uM Fe(II)/L for the red clover, and 88.71 $\pm$ 3.16 uM Fe(II)/L for the white clover. The total polyphenol content (TPC) was in the range 14.29 $\pm$ 1.19–19.20 $\pm$ 0.71 mg/L GAE and higher value was observed in the case of red clover extract (Table 1). It has been demonstrated that flower extracts of both clovers significantly differed in terms of all of the studied parameters (Table 1). There is also a strong, positive correlation between polyphenol content and reduction potential (FRAP) for the red clover extract (r = 0.9655; $p < 0.001$) and white clover extract (r = 0.9837; $p < 0.001$).

Table 1. Antioxidant potential (DPPH (2.2-diphenyl-1-picrylhydrazyl), ferric reducing antioxidant power (FRAP)), the total polyphenols content (TPC) in red and white clover flower extract (100 ppm). Different letters (a,b) in the columns represent statistically significant differences—* FDR $p < 0.05$ between particular subgroup, [a] Red Clover extract, [b] White Clover extract.

Extract	DPPH [%]	FRAP [uM Fe(II)/L]	TPC [mg/L]
Red Clover [a]	4.48 ± 0.21 *[b]	100.20 ± 4.96 *[b]	19.20 ± 0.71 *[b]
White Clover [b]	3.86 ± 0.16 *[a]	88.71 ± 3.16 *[a]	14.29 ± 1.19 *[a]

3.2. The Influence of Flower Extracts on Cytotoxicity—The Proliferation of THP-1 Cells Line

The lowest proliferation was observed for cells incubated with sodium fluoride, whereas the highest in the presence of red clover flower extract. For 10 uM NaF, there was a 27.57% decrease in the proliferation in reference to control with water, which served as the carrier for this compound. In reference to control, there was an increase in proliferation by 33.84% for the red clover and by 10.64% for the white clover. The addition of fluorine to the culture that included the extracts caused a decrease in proliferation by 19.5% for the red clover and 23.44% for the white clover, but the level was higher in comparison to cells incubated solely with toxic sodium fluoride (Table 2). The statistically significant results are presented in Table 2.

3.3. The Influence of Flower Extracts on Antioxidant Properties—THP-1 Cell Line

The antioxidant capacity of cells exposed or treated with 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical was from 13.83 to 94.09 mM. The lowest potential was observed in the case of cells incubated with sodium fluoride, whereas the highest was observed for red clover flower extract (Table 3). A significant decrease in antioxidant potential was observed in cells treated with sodium fluoride. A significant increase of antioxidant potential was recorded in cell culture with addition of the flowers' extracts. THP-1 incubation with both factors, F and flowers' extracts caused an insignificant decrease of the value in comparison to the culture with the addition of extracts (Table 3).

Superoxide dismutase activity was from 8.69 U/mL (in the culture with added sodium fluoride) to 44.54 U/mL (for red clover flower extract) (Table 3). The analysis of the studied parameters revealed decreased ability of superoxide dismutase in positive control incubated with the addition of DMSO and sodium fluoride, whereas the addition of extracts caused growth in the activity of the enzyme (Table 3).

The activity of catalase was from 46.32 nmol/min/mg to 328.81 nmol/min/mg for sodium fluoride and red clover flower extract, respectively (Table 3). It has been demonstrated that the addition of fluorine to the culture resulted in the inhibition of the enzyme, whereas the addition of clover extracts caused an increase in the activity of catalase in comparison to control. Statistically significant results for all parameters associated with antioxidant activity are presented in Table 3.

Table 2. Influence of red and white clover flower extract (100 ppm) on proliferation of macrophages incubated with NaF addition. Different letters (a–i) in the columns represent statistically significant differences—false discovery rate * FDR $p < 0.05$ between particular subgroup, n = 5. Ab—absorbance.

Sample	Negative Control [a]	Water (NaF Solvent) [b]	DMSO (Extract Solvent) [c]	Water and DMSO (NaF and Extract Solvent) [d]	NaF 10uM [e]	White Clover [f]	White Clover + NaF [g]	Red Clover [h]	Red Clover + NaF [i]
BrdU [Ab]	1.12 ± 0.03 *e	1.079 ± 0.001	1.052 ± 0.03	0.967 ± 0.03 *h	0.762 ± 0.001 *a,f,h	1.164 ± 0.004 *e,g	0.937 ± 0.001 *f,h	1.408 ± 0.001 *d,e,g	1.078 ± 0.004

Table 3. Antioxidant potential in cell cultures THP-1 with clover flower extracts (ABTS, enzyme activity—SOD, CAT). Different letters (a–i) in the columns represent statistically significant differences—* FDR $p < 0.05$ between particular subgroup, n = 5.

Sample	ABTS [mM]	SOD [U/mL]	CAT [nmol/min/mL/protein]
Negative control [a]	23.36 ± 1.93 *c,d,f,g,h,i	31.42 ± 6.38 *b,c,d	151.33 ± 8.07 *e,h
Water (NaF solvent) [b]	36.69 ± 7.79 *c,f,g,h,i	30.11 ± 12.49 *c,d,e,h	164.46 ± 35.65 *e,h
DMSO (extract solvent) [c]	67.01 ± 6.61 *a,b,e,h	11. 44 ± 2.36 *a,b,f,h,i,	203.50 ± 30.15 *e,h
DMSO + water (extract and NaF solvent) [d]	57.23 ± 15.70 *a,e,f,h,i	12.26 ± 1.18 *a,b,f,h,i,	219.38 ± 55.45 *e,h
NaF 10uM [e]	13.83 ± 3.20 *c,d,f,g,h,i	8.69 ± 1.45 *a,b,f,g,h,i	46.32 ± 9.32 *a,b,c,d,f,g,h,i
White clover [f]	91.41 ± 16,02 *a,b,d,e	32.89 ± 6.15 *c,d,e,	232.12 ± 50.34 *e,h
White clover + NaF [g]	69.40 ± 5.06 *a,b,e	24.78 ± 10.02 *e,h	157.39 ± 45.03 *e,h
Red Clover [h]	94.09 ± 24.25 *a,b,c,d,e	44.55 ± 5.57 *b,c,d,e,g	328.81 ± 68.82 *a,b,c,d,e,f,g,i
Red Clover + NaF [i]	84.37 ± 9.46 *a,b,d,e	32.89 ± 6.15 *c,d,e	227.28 ± 22.00 *e,h

4. Discussion

4.1. The Analysis of the Antioxidant Properties of Flower Extracts

Flowers are often considered only as a decorative element for meals, but in fact they have been consumed in Europe for over 500 years [8,10]. The popularity of their consumption keeps increasing thanks to the results of studies that confirm the presence of biologically active compounds and minerals within them, which have a positive influence on health [9]. Edible plants also include red and white clover plants. The role of polyphenols as protective dietary constituents with their antioxidant potential has also become an increasingly important area of research [27]. Long-term diets rich in plant polyphenols protect against chronic illnesses with a free radical basis, such as diabetes, cardiovascular diseases and cancer [28].

Our studies revealed that extracts from both clovers are characterised by antioxidant properties. In the case of *Trifolium pratense*, the percentage of DPPH inhibition was 4.48 ± 0.21%, whereas for *T. repens* the value was 3.86 ± 0.16%. The reductive potential measured by FRAP was 100.20 ± 4.96 uM Fe(II)/L for the extract acquired from the red clover, and 88.71 ± 3.16 uM Fe(II)/L for the white clover flower extract. Polyphenol content for *T. pratense* was 19.20 ± 0.71 mg/L GAE, whereas the value for *T. repens* was 14.29 ± 1.19 mg/L GAE. Both the antioxidant potential as well as polyphenol content were higher for the red clover, as demonstrated by the statistical analysis for all parameters tested ($p < 0.05$), which suggests that it is more effective in comparison to the white clover. However, these differences are not large so the white clover can also serve as a valuable yet unappreciated source of antioxidants. The results of our study are similar to those of other authors who demonstrated that the antioxidant properties of the clover are associated with its biochemical content and the high concentration of polyphenol compounds. These studies mainly concern the red clover [6,8].

Isoflavones are a group of flavonoids typical of some Leguminosae family only. In addition, in the case of isoflavones such as genistein and daidzein, their antioxidant activity, which may be of physiological importance, has also been reported. Both the red clover and the white clover are sources of flavonoids, which belong to the group of polyphenols, such as quercetin, rutin, kaempherol and trifolin [29–32]. The antioxidants content in the white clover flower extract were highest for kaempferol-3 (caffeoyldiglucoside)-7-glucoside (983.7 µg/mL), followed by *p*-coumaroyl-4-glucoside (905.6 µg/mL) and daidzein-O-sulfate (808.3 µg/mL) [33]. Large amounts of glycosyl and glycosyl malonate derivative of flavones (quercetin in leaves, quercetin and kaempferol in flowers), quercetin galactoside and its acetyl derivative, together with lower content of myricetin galactoside and isoflavones (biochanin A, formononetin in leaves and flowers) were detected in red clover [34,35].

Vlaisavljević et al. [36] demonstrated that methanol extracts at the growth phases (30 cm, 50 cm, bud) of the red clover contain rutin (0.157 $\mu g/g^{-1}$ dry mass), which belongs to the group of flavonoids and which is characterised by antioxidant properties. *Trifolium pratense* extracts were characterised by a reductive potential in the range from 376.90 mg ascorbic acid equivalent (AAE)/g to 411.40 mg AAE/g of dry mass extract depending on the plant's development stage [36]. The study conducted by Esmaeili et al. [37] revealed that the content of flavonoids in callus tissue of red clover was from 6.11 to 26.61 mg of rutin equivalents (CTE)/g dry mass. Tundis et al. [7] studied the antioxidant potential of flower extracts acquired from the red clover and the white clover originating from Calabria (Italy). The reductive activity of antioxidants (FRAP) in the white clover (*T. repens*) was equal to 44.2 µM Fe(II)/g while no activity was detected for *Trifolium pratense*. *T. repens* flowers' extract showed a good radical scavenging activity in both DPPH and ABTS tests with IC_{50} values of 10.3 and 21.4 µg/mL [7]. White clover extracts were characterised by a high concentration of rutin and quercetin in comparison to red clover extracts, which included luteolin [7]. To conclude, the different chemical composition of the species determined their antioxidant properties measured by various spectrophotometric methods.

4.2. The Influence of Flower Extracts on the Proliferation of THP-1 Cells

Studies unequivocally confirm that 10µM sodium fluoride has a negative influence on one of the parameters of cytotoxicity-proliferation. Fluorine, even in small doses (10 uM) causes changes in the activity of antioxidant enzymes, increases the synthesis of ROS, causes inflammation as well as the apoptosis and necrosis of cells, which can also have an influence on the inhibition of macrophage proliferation [4,38–42]. The addition of flower extracts to the culture with fluorine increased the proliferation of cells, regulating the whole cell cycle. Therefore, it can be concluded that the extracts demonstrated a protective effect. It is also important that no significant increase in proliferation was observed in the case of any of the two extracts in reference to control. This fact might be associated with the presence of numerous biologically active substances, including polyphenols. These are the first studies evaluating the influence of white clover and red clover flower extracts on the proliferation of THP-1 cells, demonstrated by the statistical analysis ($p < 0.05$). In the study by Booth et al. [17], scientists focused on the influence of red clover flower extracts on the proliferation of MCF-7 cells line of the oestrogen-dependant breast adenocarcinoma. An increase in proliferation in reference to control was observed, but without a cytotoxic effect [17]. However, further studies are necessary in this field because in the case of flower extracts, proliferating activity may depend on the dose and chemical composition [43].

4.3. The Influence of Flower Extracts on Antioxidant Cellular Status—THP-1 Cell Line

The antioxidant properties of extracts from both clover species were confirmed in our in vitro studies of the THP-1 cell line. Due to the fact that fluorine inhibits the activity of antioxidant enzymes and causes excessive production of oxygen radicals [4,24,25], it served as the initiator of the oxidation process. It can be concluded that in a relatively low concentration (10 uM) fluorine leads to changes in the activity of antioxidant enzymes, disrupts the effective functioning of the antioxidant apparatus, causes inflammation, apoptosis and the necrosis of cells, which is also confirmed by numerous scientific reports [1,24,38,44–47]. The cations of many elements (calcium, magnesium, manganese, aluminium) can form insoluble complexes with fluorine, which may contribute to the inhibition of the activity of enzymes that depend on those cations. Fluoride ions may also bind to functional groups of amino acids surrounding the active centre of enzymes, resulting in their inhibition [1,46].

The addition of flower extracts to the culture resulted in a change in the activity of SOD, CAT and the antioxidant potential of macrophages. On the basis of the acquired results, it can be observed that fluorine that is introduced into a culture, even with plant extracts, causes the inhibition of the studied antioxidant enzymes. We have demonstrated that the addition of a clover extract (red or white) caused a decrease in the excessive oxygen oxidation caused by fluorine [39–41]. Therefore, flower extracts protect against the toxic fluoride in macrophages of the THP1 line, demonstrated by the statistical analysis ($p < 0.05$). Our study confirms the findings of other authors who revealed the antioxidant properties of the red clover. The majority of scientific reports concerning the biological activity of *Trifolium* species contain results of in vitro and in vivo studies on *Trifoliumpratense* [6,37]. Mu et al. also showed that *Trifolium pratense* showed recovery of liver antioxidant enzymes and lipid peroxidation [48]. In a study on rats that were supplied with bisphenol A (BPA) and a water-ethanol extract from the red clover, it was observed that the extract prevented the influence of BPA on the peroxidation of lipids and had a protective effect [49]. Water extracts of the white clover, rich in phenolic compounds, had an influence on the reduction of lipid peroxidation and the normalisation of the total reduce glutathione in the liver [33].

The red clover and the white clover contain numerous elements, such as calcium, chromium, iron, magnesium, manganese, copper, selenium and zinc [50]. The antioxidant mechanism of the extract may be associated with the content of manganese, zinc and copper—the cofactors for superoxide dismutase, which unblocked antioxidant enzymes and increased their activity. Moreover, numerous polyphenol compounds present in the extracts might have caused an increase in the activity of antioxidant enzymes (super-

oxide dismutase, catalase, glutathione peroxidase) and contribute to the increase in the concentration of low molecular weight antioxidants (ascorbic acid, a-tocopherol) [51–53].

It is also worth highlighting that the antioxidant properties of extracts of the less-known, but readily available clover studied on cell lines show a similar or stronger positive effect on health in comparison to other, often more popular plants, e.g., *Camellia* extract or *Castanea crenata* [54,55].

5. Conclusions

Food and nutraceuticals are rich in important polyphenolic compounds which are the best antioxidants. *Trifolium repens* and *Trifolium pratense* flower extract proved to have a protective effect against oxidative stress induced by fluoride in THP-1 cells, which was demonstrated for the first time. The antioxidant properties of extracts of the under-appreciated, but readily available clover, both red and white, show a similar or stronger effect in comparison to other known plant resources. Therefore, they can serve as a valuable, readily available and inexpensive source of antioxidants, which are necessary both in the prophylaxis as well as the support of treatment of numerous diseases of civilisation with a free radical basis.

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