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Genotoxic properties of *Betonica officinalis, Gratiola officinalis, Vincetoxicum luteum* and *Vincetoxicum hirundinaria* extracts

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ABSTRACT

Genotoxicity of *B. officinalis, G. officinalis, V. luteum* and *V. hirundinaria* extracts, which demonstrated strong antioxidant capacity, was tested using chromosome aberration, sister chromatid exchange (SCE), cytokinesis-block micronucleus and alkaline single-cell gel electrophoresis (comet) assays in human lymphocytes *in vitro* and Ames *Salmonella*/microsome test. All tested extracts were not mutagenic in *S. typhimurium* strains TA98 and TA100 with and without metabolic activation and did not induce chromosome aberrations in human lymphocytes *in vitro*. Extract from *G. officinalis* was the only one, which induced significant increase in micronuclei, indicating possible aneugenic effect. All investigated plant extracts induced DNA damage evaluated by the comet assay, while *B. officinalis* and *V. luteum* extracts induced slight increase in SCE values. The determined variation in response might be due to the plant extract tested and donor susceptibility.

1. Introduction

Medicinal plants were the main remedies until modern times and significantly influenced development of many synthetic pharmaceuticals, while nowadays their importance has gained new implications due to the growing interest in functional foods and nutraceuticals. Therefore, comprehensive toxicological evaluation of such species is among the most important issues for their valorization (Shipkowski et al., 2018). This study evaluated genotoxicity and mutagenicity of the extracts of *Betonica officinalis* L. (syn. *Stachys officinalis* (L.) Trevis. ex Briq.), *Gratiola officinalis* L., *Vincetoxicum luteum* L. and *Vincetoxicum hirundinaria* L., which demonstrated strong antioxidant potential and may be considered as promising sources of bioactive compounds (Šliumpaitė et al., 2013a,b,c).

The plants of *Stachys* have been used in folk medicine to treat various diseases and health disorders. Wood betony (a common name of *B. officinalis*) has been used in some countries in the form of tea and dried leaves for antibacterial purposes, against headache, nervous tension, anxiety, menopausal problems, and as a tobacco snuff (Gören, 2014). Šliumpaitė et al. (2013a) reported chlorogenic acid, phenylethanoid glycosides and hydroxycinnamic acid derivatives as the main antioxidants in wood betony extracts. Háznagy-Radnai et al. (2006b) suggested tannins as the main components responsible for the antioxidant effect of methanolic plant extract, while harpagoside, harpagide, and acetylharpagide were the main iridoids. *S. officinalis* methanolic extract lowered the degree of lipid peroxidation induced by hydroxyl radical (Matkowski and Piotrowska, 2006), various *Stachys* spp. demonstrated radical scavenging activity (Vundać et al., 2007). Picker et al. (2010) reported anti-inflammatory properties of *B. officinalis* dichloromethane extracts, derived fractions, and isolated pure compounds. Iridoids isolated from the selected *Stachys* spp. showed cytotoxic activity on the HeLa A431 and MCF7 cell lines (Háznagy-Radnai et al., 2008). *S. officinalis* aqueous extract inhibited the development of carrageenan-induced inflammatory oedema in male Sprague-Dawley rats; iridoids aucubin and harpagoside demonstrated high dose-dependent antiphlogistic effects (Háznagy-Radnai et al., 2012). More recently, Romanian researchers showed that hydroethanolic extract of wood betony inhibited COX-1 and denatured protein, thus acting as natural anti-inflammatory and wound healing agent (Paun et al., 2017). Microfiltrated fraction of *B. officinalis* hydroethanolic extract, containing chlorogenic, caffeic, rosmarinic and ellagic acids, inhibited α-amylase and β-glucosidase and was suggested for developing anti-diabetic preparations (Paun et al., 2016). Later Paun et al. (2018) reported that the nanofiltrated extracts had high DPPH$^{\bullet}$-scavenging

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capacity and inhibited lipoxygenase, COX-1 and COX-2. Moreover, anti-inflammatory effect was supported by the *in vivo* studies with male rats.

Common hedge hyssop (*G. officinalis* L., Plantaginaceae) has been used for treating liver diseases, visceral obstructions, skin diseases, menstrual disorders, gout and for expelling parasitic worms. It was also known for affecting nervous system and possessing some effects on gastrointestinal conditions (Šliumpaitė et al., 2013b). Methanol and acetone extracts of *G. officinalis* effectively scavenged $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, while phenylpropanoid glycosides were determined as the main antioxidants (Šliumpaitė et al., 2013b). Various health benefits were reported for flavonoid-rich *G. officinalis* extract: (i) dose-dependent antitumor and apoptotic activity against kidney cancer Caki-1 and Sn12c cultures (Polukonova et al., 2018); (ii) MDA concentration and lipid hydroperoxide reducing and vitamin E increasing effects in the blood serum of rats with transplanted tumors (Navolokin et al., 2017a); (iii) pronounced pathomorphism in kidney cancer (Navolokin et al., 2017b); (iv) normalizing myelocytic germ parameters in bone marrow of tumor-bearing rats and increasing lymphocyte percent effects in white blood cell count of blood and myelogram (Navolokin et al., 2017c). Other bioactivities reported for *G officinalis* preparations include inhibition of dioxidin-induced lipid peroxidation in blood of rats (Durnova et al., 2015), bactericidal effects (Navolokin et al., 2015), antimicrobial (Polukonova et al., 2015) and membranoprotective properties (Tkachenko et al., 2015). Ahmad et al. (2012) reported biological (Brine Shrimp Bioassay, Insecticidal and Phytotoxicity/Cytotoxic) and neuropharmacological (Head dip, Open field Forced swimming test, Sodium pentothal induced sleep) activities of *G. officinalis* crude extract and its hexane, chloroform, ethyl acetate, butanol and aqueous fractions.

The name *Vincetoxicum* (in Latin meaning 'conqueror of poison') derives from the traditional use of this plant as an antidote to poisons. *V. hirundinaria* (Apocynaceae) is a long-lived herbaceous perennial plant; its common name white swallow-wort comes from *hirundo* meaning swallow (Nature Gate, 2019). It is a venomous plant traditionally used in treating diseases although the reports on its chemical composition and bioactivities are rather scarce. Laukkanen et al. (2012) reported 218–238 mg/g of lipophilic compounds, 8.5–23.7 mg/g flavonoids, 3.2–5.0 mg/g chlorogenic acid, 0.61–1.2 mg/g catechin derivatives and 0.46–0.97 mg/g antofin in the leaves. *V. hirundinaria* was shown as effective agent against *Spodoptera littoralis* larvae (Pavela, 2010, 2011). It is included into homeopathic preparation Engystol, which significantly increased the percentage of interferon-gamma producing lymphocytes (Enbergs, 2006) and reduced onset of experimental syncytial virus-induced respiratory inflammation in mice (Wronski et al., 2018). However, it should be noted that Engystol is a homeopathic product, which is in principle different comparing with plant extracts used in ethnopharmacology.

Yellow swallow-wort (*Vincetoxicum luteum*, L., or *Vincetoxicum inamoenum*, Maxim.; Apocynaceae) is a herbaceous twining perennial plant (Sheeley and Raynal, 1996). Its rhizomes, leaves and dry seeds have been used for various medicinal purposes, e.g. to treat neurosis and malaria, rupture, internal fever, scrofula and scabies, accelerating wound healing as well as for diuretic, laxative and emetic effects (Fang et al., 1995). It should be noted that swallow-wort is a poisonous plant; a large dose affects nervous system and heart.

Our previous studies demonstrated significant antioxidant potential of woody betony, hedge hyssop and swallow-wort extracts (Šliumpaitė et al., 2013a,b,c), while screening their phytochemical composition revealed the presence of various phytochemicals, mainly polyphenolic compounds. However, to the best of our knowledge, genotoxicity of these herbs have not been evaluated until now. To fill this gap the aim of our study was to evaluate genotoxic potential of *B. officinalis*, *G. officinalis*, *V. luteum* and *V. hirundinaria* extracts. For this purpose chromosome aberration, sister chromatid exchange, cytokinesis-block micronucleus, alkaline single-cell gel electrophoresis (comet) assays in human lymphocytes *in vitro* and Ames *Salmonella*/microsome test were used. The results obtained may assist in further valorization of the plants for their wider applications.

2. Materials and methods

2.1. Plant material and preparation of extracts

All plants were grown in the collection of medicinal plants of Kaunas Botanical Garden at Vytautas Magnus University (Kaunas, Lithuania). After harvesting they were sorted out, dried at 30 °C and powdered in an ultra-centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve. Five g of powdered sample were extracted with 120 mL of acetone (b.p. 57 °C, OBR PR, Plock, Poland) using 3 × 40 min extraction cycles at 70 °C in an automatic extractor IKA Werke RET control-visc IKAMAG safety control (Staufen, Germany). The residues were dried and re-extracted with 120 mL of methanol (b.p. 65 °C, Lachema, Brno, Czech Republic) also applying 3 × 40 min cycles. The extracts were concentrated in a rotary evaporator Büchi, R-114 (Donau, Flavil, Switzerland) at approximately 40 °C and finally dried by a flow of nitrogen. The extracts were stored at −20 °C prior to further analysis. Total phenolic content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC) of extracts was determined by the methods of Singleton and Rossi (1965) and Re et al. (1999), respectively. Detailed description of the procedures may be found elsewhere (Šliumpaitė et al., 2013a,b,c).

2.2. Chemicals and reagents

Methanol, acetic acid, potassium chloride, sodium chloride, sodium citrate, Giemsa stain, Sorensen's buffer, DMSO were obtained from Merck (Germany) and doxorubicin from Teva Pharma B.V. (The Netherlands). All other reagents for the cytogenetic tests and comet assay were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). *S. typhimurium* tester strains, S9 fractions and cofactor reagents, culture media and all other products for the Ames test were from Moltox (Molecular Toxicology, Inc., USA).

2.3. The Ames salmonella/microsome test

Standard plate incorporation method using histidine-dependent *Salmonella typhimurium* strains TA98 and TA100 (Mortelmans and Zeiger, 2000) was used to assess the mutagenicity in the presence and in the absence of *in vitro* metabolic activation system (Aroclor-1254 induced male Sprague Dawley rat liver post-mitochondrial S9 fraction). Plant extracts were dissolved in ethanol and then diluted to the desired concentration in sterile distilled water. Concurrent positive and vehicle (ethanol) controls were used in the study. The standard mutagens used as positive controls were daunomycin for TA98 strain and sodium azide for TA100 strain without metabolic activation and 2-aminoanthracene for both TA98 and TA100 strains with metabolic activation. The plates were incubated at 37 °C for 48 h and revertant colonies were counted using automatic Sorcerer colony counter (Perceptive Instruments, UK). The experiments were analysed in triplicate and the results are presented as the mean of the three plates with standard deviation. The criteria employed to interpret the results of Ames test as positive were similar to those used in regulatory guidelines (OECD guidelines, 1997). For a test compound to be considered positive, it had to produce at least a 2-fold increase in the mean number of revertant colonies per plate over the mean revertant colonies per plate of the appropriate vehicle control. In addition, this increase had to be accompanied by a dose response to increasing concentrations of the test substance.

2.4. Blood sampling and cell culture procedures

Four healthy non-smoking volunteers donated blood samples for this study: donors A, B and C females, 53, 24 and 22 years old,

respectively; donor D – 26 years old male. Blood samples were taken into heparinized vacutainer tubes (Becton-Dickinson, USA) immediately before the conducting the test. The cultures were made in RPMI 1640 medium supplemented with 12% heat-inactivated newborn calf serum, 7.8 μg/mL phytohemagglutinin, 40 μg/mL gentamycin. In addition, cultures assigned to the chromosome aberration (CA) and sister chromatid exchange (SCE) assay were supplemented with 10 μg/mL 5-bromo-2′-deoxyuridine for the entire culture period. They were incubated at 37 °C for 72 h.

Plant extracts were dissolved in ethanol and then diluted with RPMI 1640 medium to the desired concentration. Working solutions were made just before treatment. Two parallel cultures were used for each concentration of the extract tested. Ethanol in a final concentration of 7.5 μL/mL was used as a vehicle control. Ethanol concentration in the experimental series did not exceed this concentration. Two cultures were left untreated and served as a blank control.

2.5. Chromosome aberration (CA) and sister chromatid exchange (SCE) assay

Blood samples obtained from donors A and D were used in this experiment. Treatment with plant extracts was carried out 48 h after culture initiation and lasted for the period of 24 h. Cultures treated with methyl methanesulfonate (MMS) at a final concentration of 25 μg/mL were used as a positive control. The cultures were treated with colchicine (0.6 μg/mL) for the last 3 h of incubation. The cells were harvested using a routine protocol, including hypotonic treatment with 0.075 M KCl for 25 min and three periods of fixation in methanol:acetic acid (3:1, v/v). Air-dried slides were prepared and differentially stained by the fluorescence plus Giemsa technique (Lazutka, 1991). Briefly, the slides were stained for 10 min with 10 μg/mL of Hoechst 33258 dye (dissolved in 0.07 M Sorensen's buffer, pH 6.8), then rinsed, mounted with citrate buffer (pH 8.5), covered with cover slips and exposed to UV light (400 W mercury lamp at a distance of 15 cm) for 6–7 min. Slides were then rinsed and stained with 5% Giemsa (Merck, Germany) for 3–4 min. All slides were coded and scored at 1000 × magnification on a light microscope (Nikon, Japan). No less than 100 first-division metaphases per concentration were analysed for chromosome aberrations. The CA were recorded according to An International System for Human Cytogenetic Nomenclature (Shaffer and Tommerup, 2005). Aberrations were scored as individual types (chromatid and chromosome type breaks and exchanges), but for statistical analysis only total CA per 100 cells was used. SCEs were scored in 50 s-division metaphases per treatment. Cell replicative kinetics was determined by means of replicative index (RI = $[M_1 + 2M_2 + 3M_3]/N$, where M_1, M_2, M_3 are the numbers of cells that had undergone one, two or three cycles of replication, and N is a total number of cells scored). Two hundred cells were scored to determine RI.

2.6. Cytokinesis-block micronucleus assay

Donors A, C and D donated their blood samples for this part of the study. Cytochalasin B at a final concentration of 6 μg/mL was added to the cultures 44 h after phytohemagglutinin stimulation to arrest cytokinesis and to obtain binucleated cells. Treatment with extracts was carried out 24 h after culture initiation and lasted for the period of 48 h. Cultures treated with doxorubicin at a final concentration of 0.02 μg/mL were used as positive control. At 72 h, the cells were subjected to a cold hypotonic treatment with 0.075 M KCl, then centrifuged immediately and fixed three times with cold methanol: acetic acid (5:1, v/v; first portion of fixative was diluted with equal volume of 0.9% NaCl). The cells smeared on a precleaned microscope slides, air-dried and stained with 5% Giemsa solution in Sorensen's buffer pH 6.8. All slides were coded and analysed at 1000x magnification. One thousand of cytochalasin B-blocked binucleated cells were analysed per concentration for the presence of micronuclei (MN) using standard scoring criteria (Fenech, 2007). The nuclear division index (NDI) was used for measuring cell proliferation kinetics and was calculated by scoring cells with 1, 2, 3 or 4 nuclei and using the formula: NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1, M2, M3 and M4 represent the number of cells with 1, 2, 3 or 4 nuclei and N is the total number of cells scored. Five hundred cells were scored to determine NDI.

2.7. Comet assay

Peripheral blood was obtained from donors A and B. The comet assay was carried out using the procedure of Singh et al. (1988) with minor modifications. Briefly, lymphocytes were isolated by Lymphoprep density gradient centrifugation according to the manufacturer's instructions (Axis-Shield, Norway). Following isolation, the cells were resuspended in RPMI 1640 medium, adjusted to about 2×10^5 lymphocytes/mL and incubated with plant extracts (dose range from 50 to 250 μg/mL) for 1 h at 37 °C in a 5% CO_2. Ethanol (0.3% v/v) served as the vehicle control, and H_2O_2 at 20 μM concentration was used as the positive control. After treatment, 40 μL of cell suspension was mixed with 40 μL of 1% low melting point agarose (LMPA) and spread onto the slides precoated with 1% normal melting point agarose. The agarose was allowed to solidify, and afterwards covered with a third layer of 0.5% LMPA. After coverslips removal, lysis was carried out for 1.5 h at 4 °C in cold freshly prepared solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, with 1% Triton X-100 and 10% DMSO added just before use, pH 10). The microscope slides were then placed into ice-cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 20 min and then electrophoresis was performed at 17 V and 300 mA for 30 min. Finally, the slides were neutralized with Tris buffer (0.4 M Tris, pH 7.5), and stained with ethidium bromide (20 μg/mL). The coded slides were examined at 400x magnification. Image capture and analysis were performed using Lucia-Comet image analysis system (Laboratory Imaging, Prague, Czech Republic). In each experiment, 100 comets were randomly selected to evaluate DNA damage for each concentration of the tested extract. The percentage of DNA in the comet tail (% TDNA) was used as DNA damage parameter.

2.8. Statistical analysis

Statistical analysis of genotoxicity results was performed using the SPSS (Version 19) package (SPSS, Inc., Chicago, IL, USA). Statistical tests were chosen according to the nature of the data analysed. The Student's two sided *t*-test was applied for MN, CA, SCE and comet assay results, comparing the different treatment groups, and z test for NDI and RI analysis (Lazutka, 1991). Dose–response relationships were determined by Pearson's correlation. P value < 0.05 was regarded as statistically significant.

3. Results and discussion

3.1. Characterisation of extracts

Bioactivities of plant origin preparations very often are related to the presence and concentration of polyphenolic compounds and antioxidant capacity. The values of total phenolic content (TPC) measured with Folin-Ciocalteu's reagent and Trolox Equivalent Antioxidant Capacity of extracts measured by ABTS radical cation decolourisation assay are presented in Table 1. It is interesting noting that the yields obtained with medium polarity aprotic solvent acetone and high polarity protic solvent methanol remarkably differed between the analysed plants. Thus, the yield of methanol extract from *B. officinalis* was more than 3-fold higher than that of acetone, the yield from *G. officinalis* almost similar in case of both solvents, whereas methanol yields from *Vincetoxicum* plants were more than 2-fold lower that acetone extraction yields. It indicates on the presence of different amounts of higher and lower polarity compounds in the medicinal plants.

Table 1
Extraction yields (%, w/w) total amount of phenolic compounds (TPC, mg GAE/g dry extract) and Trolox equivalent antioxidant capacity (TEAC, μM/g dry extract) of plant extracts.

Solvent	*B. officinalis*			*G. officinalis*			*V. luteum*			*V. hirundinaria*		
	Yield	TPC	TE	Yield	TPC	TEAC	Yield	TPC	TEAC	Yield	TPC	TEAC
Methanol	18.6	61.2	714	14.4	14.0	1093	9.2	86.0	674	8.7	93.1	698
Acetone	5.6	82.3	558	14.3	10.0	746	24.2	131.8	1026	21.6	127.4	977

Remarkably lower TPC values were determined for common hedge hyssop extracts comparing to other plants; however, the differences in TEAC values were less pronounced. In general, all prepared extracts effectively scavenged ABTS radical cation; TEAC values were from 558 to 1093 μM TE/g, which indicates that 1 g of extracts possessed antioxidant capacity equal to 0.14–0.27 mg synthetic antioxidant Trolox.

According to the on-line HPLC/UV/DPPH$^{\bullet}$-scavenging results, catechin, gallic, chlorogenic, caffeic, ferulic and ellagic acids were the main antioxidants in *B. officinalis* extracts (Šliumpaitė et al., 2013a); 3 tentatively identified phenylpropanoid glycosides and 5 detected but not identified compounds in *G. officinalis* extracts (Šliumpaitė et al., 2013b), and chlorogenic acid, isoquercitrin, apigenin-7-*O*-glucoside, caffeic acid and 3 detected but not identified compounds in *Vincetoxicum* extracts (Šliumpaitė et al., 2013c).

3.2. Genotoxicity evaluation

Evaluation of genotoxic properties is an essential step of the safety assessment of substances intended to use as pharmaceuticals or food additives (ICH S22(R1), 2011). Usually such assessment is being done using test battery of three tests – the bacterial reverse mutation assay, the *in vitro* mammalian test, and the *in vivo* mammalian test. However, considering a reduction of animal use, as well as reduction of costs, two-stage genotoxicity testing strategy has been proposed (COM, 2011): initial testing is based upon the *in vitro* tests, while next stage uses different *in vivo* mammalian tests. The similar approaches have been discussed recently in great details (Corvi and Madia, 2017).

Here we report the results of initial genotoxicity testing of extracts from medicinal herbs *B. officinalis, G. officinalis*, *V. luteum* and *V. hirundinaria.* Our tests cover the three main endpoints of genotoxicity: gene mutations (the Ames test), structural (micronucleus test and chromosome aberration tests) and numerical (micronucleus test) chromosome mutations. We also used two additional tests reflecting mainly primary DNA damage and/or DNA repair (sister-chromatid exchange test and Comet assay).

3.2.1. Salmonella/microsome test

The Ames *S. typhimurium* reverse mutation test is commonly used to screen mutagenicity, in particular, point mutation-inducing activity of chemical compounds and mixtures. We assayed the mutagenic activities of plant extracts in the *S. typhimurium* tester strains TA98 (detects frameshift mutations) and TA100 (detects base-pair-substitution mutations) in the presence and in the absence of *in vitro* metabolic activation system (S9 fraction). The mutagenic index (MI) was calculated for each tested concentration. MI corresponded to the mean number of revertant colonies per plate containing the test extract divided by the mean number of revertant colonies per plate containing the vehicle control.

All data are presented in Tables 2 and 3. None of the tested extracts was mutagenic to *S. typhimurium* strain TA100. Only extracts of *B. officinalis* at 500 μg/plate with and without metabolic activation and *G. officinalis* at 100 μg/plate without metabolic activation produced a 2-fold increase in the mean revertants per plate in TA98 strain. However, no dose-response relationship was observed. All other extracts did not increase the number of revertant colonies per plate over the mean revertants per plate of the vehicle control. Thus, under the conditions of this study all investigated extracts showed negative results in the Ames test, indicating that they do not produce reverse mutations in *S. typhimurium* TA98 and TA100 strains.

3.2.2. Chromosome aberration and sister chromatid exchange analysis

The *in vitro* chromosome aberration assay is a key testing method for genotoxicity assessment. It is used to identify agents that cause structural chromosomal abnormalities, such as breaks and exchanges, which may be of two types, chromosome and chromatid.

Lymphocytes from two donors (donors A and D) were used for this part of the study. Genotoxicity of *B. officinalis* extract was studied in the range of concentrations from 50 to 200 μg/mL. Extracts prepared from *G. officinalis, V. hirundinaria* and *V. luteum* were cytotoxic at these concentrations and therefore investigated at lower concentrations ranging from 12.5 to 50 μg/mL. However, 50 μg/mL concentration of *G. officinalis, V. hirundinaria* and *V. luteum* extracts was found to be cytotoxic for donor D lymphocytes.

In human lymphocytes in *vitro,* none of the tested extracts induced significant increase in chromosome aberrations (Table 4). In the case of SCE, the slight increase in SCE values was observed in cultures treated with extracts from *B. officinalis* and *V. luteum* (Table 5). Dose-response relationship was linear for *B. officinalis* extract in both donors: $y = 7.16 + 0.09x$ ($r = 0.96$, $P = 0.009$, donor A) and $y = 6.95 + 0.01x$ ($r = 0.97$, $P = 0.006$, donor D), where y is the number of SCE per cell, x is the concentration of extract. The established dose-response relationship for *V. luteum* extract was $y = 8.44 + 0.05x$ ($r = 0.99$, $p = 0.002$) in donor A lymphocytes, though no significant increase in SCEs in donor D lymphocytes was determined. *G. officinalis* and *V. hirundinaria* extracts were not genotoxic for the lymphocytes of both donors. All tested extracts clearly inhibited cell replicative kinetics in donor A lymphocytes. A dose-response relationships were linear for all extracts and may be described by equations: $y = 2.50–0.01x$ ($r = -0.98$, $P = 0.005$) for *B. officinalis*, $y = 2.43–0.02x$ ($r = -0.93$, $P = 0.023$) for *G. officinalis,* $y = 2.12–0.02x$ ($r = -0.93$, $P = 0.022$) for *V. hirundinaria* and $y = 2.12–0.02x$ ($r = -0.90$, $P = 0.038$) for *V. luteum* extract, where y is the replication index (RI) value and x is the concentration of extract (μg/mL). Both *Vincetoxicum sp.* extracts inhibited cell replicative kinetics in a dose-dependent manner in donor D lymphocytes also. Dose dependency was linear and may be described by equations $y = 2.15–0.01x$ ($r = -0.98$, $P = 0.017$) and $y = 2.17–0.01x$ ($r = -0.99$, $P = 0.006$) for *V. hirundinaria* and *V.luteum,* respectively.

In summary, all four tested plant extracts did not induce CAs in human lymphocytes in *vitro*, but two of them (*B. officinalis* and *V. luteum* extracts) revealed to be more potent inducers of SCEs. All four tested extracts inhibited cell replicative kinetics that may indicate cytotoxicity of extracts under the conditions of the present study.

3.2.3. Micronuclei assay

Micronuclei originate from acentric chromosome or chromatid fragments or whole chromosomes that fail to be included into the daughter nuclei during mitosis because they did not attach to the spindle during the segregation at anaphase. Thus, micronuclei may result from clastogenic (chromosome breaking; DNA as target) or aneugenic (genome mutations; spindle disturbances) mechanisms (Albertini et al., 2000).

Lymphocytes from three donors (donors A, D and C) were used for

Table 2
The numbers of revertant colonies induced by *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts in *S. typhimurium* TA98 strain with and without metabolic activation (S9 mix).

Treatment (plant extract)	Dose, μg/plate	Without S9 mix		With S9 mix	
		Revertant colonies/plate, mean ± S.D.	MI[a]	Revertant colonies/plate, mean ± S.D.	MI[a]
Blank control		54.5 ± 12.1		40.5 ± 6.3	
Vehicle, μL[b]	100	37.5 ± 4.9		36.0 ± 4.2	
Daunomycin	6	1279.0 ± 58.0			
2-Aminoanthracene	10			823.0 ± 132.9	
B. officinalis	100	48.3 ± 14.1	1.28	71.3 ± 5.1	1.98
	200	59.3 ± 7.1	1.58	63.3 ± 9.3	1.76
	300	46.0 ± 6.1	1.23	66.0 ± 4.4	1.83
	500	76.7 ± 29.7	2.05	74.0 ± 28.2	2.06
G. officinalis	100	78.0 ± 23.9	2.08	51.3 ± 19.8	1.43
	200	31.0 ± 9.5	0.83	45.5 ± 0.7	1.26
	300	39.0 ± 6.0	1.04	40.0 ± 4.2	1.11
	500	39.3 ± 9.3	1.05	37.3 ± 4.9	1.04
V. hirundinaria	100	44.0 ± 12.3	1.17	35.0 ± 7.9	0.97
	200	36.0 ± 11.3	0.96	46.3 ± 11.6	1.29
	300	62.7 ± 21.0	1.67	53.3 ± 3.8	1.48
	500	49.7 ± 19.4	1.33	44.0 ± 21.0	1.22
V. luteum	100	55.7 ± 4.9	1.49	48.0 ± 4.0	1.33
	200	34.3 ± 5.6	0.91	42.7 ± 12.5	1.19
	300	57.3 ± 21.7	1.53	46.0 ± 8.5	1.28
	500	39.0 ± 7.1	1.04	48.7 ± 4.7	1.35

[a] MI, mutagenicity index; number of revertant colonies per treated plate/number of revertant colonies per vehicle control plate.
[b] Ethanol:water (1:1).

this part of the study. Genotoxicity of *B. officinalis* extract was studied in the range of concentrations from 50 to 200 μg/mL. Extracts prepared from *G. officinalis, V. hirundinaria* and *V. luteum* were cytotoxic at these concentrations. Therefore, these extracts were tested at lower concentrations: *G. officinalis* in the concentrations ranging from 12.5 to 50 μg/mL, and *V. hirundinaria* and *V. luteum* in the range from 1.56 to 12.5 μg/mL.

Frequencies of micronucleated cytokinesis-blocked lymphocytes (MNCB) and nuclear division index (NDI) values in human lymphocytes treated with *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts are presented in Table 6. Positive control (doxorubicin, 0.02 μg/mL) significantly increased the frequency of micronucleated

Table 3
The numbers of revertant colonies induced by *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts in *S. typhimurium* TA100 strain with and without metabolic activation (S9 mix).

Treatment (plant extract)	Dose, μg/plate	Without S9 mix		With S9 mix	
		Revertant colonies/plate, mean ± S.D.	MI[a]	Revertant colonies/plate, mean ± S.D.	MI[a]
Blank control		80.5 ± 9.2		71.0 ± 7.8	
Vehicle, μL[b]	100	69.5 ± 3.5		72.5 ± 2.1	
Sodium azide	1.5	570.0 ± 74.9			
2-Aminoanthracene	10			1232.5 ± 190.6	
B. officinalis	100	90.7 ± 8.9	1.31	77.3 ± 4.7	1.07
	200	94.7 ± 28.0	1.36	77.3 ± 10.1	1.07
	300	70.3 ± 13.1	1.01	83.7 ± 10.7	1.15
	500	108.0 ± 21.4	1.55	82.0 ± 27.8	1.13
G. officinalis	100	74.3 ± 11.5	1.07	69.0 ± 7.0	0.95
	200	110.7 ± 23.15	1.59	77.3 ± 5.13	1.07
	300	83.0 ± 14.4	1.19	72.7 ± 21.2	1.00
	500	77.7 ± 13.9	1.12	73.3 ± 4.9	1.01
V. hirundinaria	100	128.3 ± 24.5	1.85	86.3 ± 23.1	1.19
	200	99.7 ± 24.0	1.43	62.0 ± 9.64	0.86
	300	98.0 ± 10.2	1.41	70.7 ± 14.6	0.99
	500	91.3 ± 49.3	1.31	69.3 ± 6.4	0.96
V. luteum	100	75.7 ± 19.5	1.09	63.0 ± 4.0	0.86
	200	85.3 ± 16.7	1.23	81.3 ± 3.5	1.12
	300	101.3 ± 11.9	1.46	74.0 ± 10.4	1.02
	500	64.7 ± 7.6	0.93	100.0 ± 2.0	1.38

[a] MI, mutagenicity index; number of revertant colonies per treated plate/number of revertant colonies per vehicle control plate.
[b] Ethanol:water (1:1).

Table 4
Frequencies of chromosome aberrations (CAs) in human lymphocytes treated *in vitro* with *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts.

Treatment (plant extracts)	Concent-ration (μg/mL)	Donor A		Donor D	
		CA per 100 cells (mean ± S.E.M.)	Aberrations ct/cs[a]	CA per 100 cells (mean ± S.E.M.)	Aberrations ct/cs[a]
Blank		2.5 ± 1.6	2/3	2.0 ± 1.4	2/0
Ethanol	7 μL/mL	2.5 ± 1.6	2/3	2.0 ± 1.4	2/0
MMS	0.02 μL/mL	12.0 ± 3.2	9/3	22.0 ± 4.1	18/4
B. officinalis	50	3.0 ± 1.7	1/2	3.0 ± 1.7	2/1
	100	5.0 ± 2.2	3/2	4.0 ± 2.0	2/2
	150	5.0 ± 2.2	3/2	5.0 ± 2.2	3/2
	200	6.0 ± 2.4	5/1	6.0 ± 2.4	5/1
Blank		2.0 ± 1.4	1/1	3.0 ± 1.7	3/0
Ethanol	7 μL/mL	2.0 ± 1.4	1/1	4.0 ± 2.0	3/1
G. officinalis	12.5	2.0 ± 1.4	1/1	5.0 ± 2.2	3/2
	25	5.0 ± 2.2	4/1	6.0 ± 2.4	5/1
	37.5	5.0 ± 2.2	4/1	4.0 ± 2.0	2/2
	50	4.0 ± 2.2	4/1	cytotox	cytotox
V. hirundinaria	12.5	3.0 ± 1.7	2/1	6.0 ± 2.4	3/3
	25	3.0 ± 1.7	1/2	5.0 ± 2.2	4/1
	37.5	5.0 ± 2.2	4/1	8.3 ± 3.0	6/1
	50	5.0 ± 2.2	4/0	cytotox	cytotox
V. luteum	12.5	3.0 ± 1.7	1/2	4.0 ± 2.0	2/2
	25	2.0 ± 1.4	2/0	4.4 ± 2.1	4/0
	37.5	5.0 ± 2.2	3/2	5.8 ± 3.2	1/2
	50	3.0 ± 1.7	2/1	cytotox	cytotox

[a] ct – chromatid type aberrations, cs – chromosome type aberrations.

cells in all donors (25.0–51.0‰). No statistically significant increase of MNCB when compared with the vehicle controls was observed after treatment of human lymphocytes with *B. officinalis, V. hirundinaria* and *V. luteum* extracts. *G. officinalis* extract induced statistically significant increase of MNCB in donor A lymphocytes, no dose-dependent relationship was observed. Increased frequency of MNCB was also

Table 5
Effects of *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts on the frequency of sister chromatid exchange (SCE) and replication index (RI) values in human lymphocyte cultures *in vitro*.

Treatment (plant extracts)	Concent-ration (μg/mL)	Donor A		Donor D	
		SCE/cell (mean ± S.E.M)	RI (mean ± S.E.M.)	SCE/cell (mean ± S.E.M)	RI (mean ± S.E.M.)
Blank		7.06 ± 0.74	2.67 ± 0.04	6.76 ± 0.41	2.46 ± 0.05
Ethanol	7.5 μL/mL	7.44 ± 0.45	2.55 ± 0.05	6.70 ± 0.36	2.41 ± 0.05
MMS	0.02 μL/ml	31.72 ± 1.16[a]	2.21 ± 0.05	46.67 ± 1.87[a]	2.10 ± 0.06[a]
B. officinalis	50	8.20 ± 0.43	2.27 ± 0.06[a]	7.86 ± 0.49[a]	2.45 ± 0.06
	100	8.62 ± 0.47	1.91 ± 0.06[a]	8.04 ± 0.43[a]	2.43 ± 0.06
	150	9.60 ± 0.49[a]	1.90 ± 0.06[a]	8.58 ± 0.46[a]	2.31 ± 0.06
	200	11.52 ± 0.54[a]	1.56 ± 0.05[a]	9.10 ± 0.48[a]	2.36 ± 0.06
Blank		8.56 ± 0.53	2.49 ± 0.05	7.16 ± 0.32	2.35 ± 0.06
Ethanol	7.5 μL/mL	8.46 ± 0.53	2.30 ± 0.06	7.64 ± 0.55	2.18 ± 0.06
G. officinalis	12.5	7.94 ± 0.50	2.30 ± 0.06	7.16 ± 0.42	2.37 ± 0.06[a]
	25	8.46 ± 0.55	2.22 ± 0.06	7.06 ± 0.45	2.42 ± 0.06[a]
	37.5	8.64 ± 0.62	1.77 ± 0.06[a]	7.10 ± 0.37	2.22 ± 0.06
	50	8.92 ± 0.41	1.58 ± 0.05[a]	cytotox	cytotox
V. hirundinaria	12.5	10.36 ± 0.48[a]	1.67 ± 0.05[a]	8.00 ± 0.55	1.96 ± 0.06[a]
	25	8.74 ± 0.58	1.58 ± 0.05[a]	7.50 ± 0.42	1.89 ± 0.06[a]
	37.5	7.90 ± 0.40	1.32 ± 0.04[a]	7.30 ± 0.51	1.73 ± 0.06[a]
	50	7.92 ± 0.43	1.25 ± 0.03[a]	cytotox	cytotox
V. luteum	12.5	8.98 ± 0.41	1.80 ± 0.05[a]	7.20 ± 0.42	1.98 ± 0.06[a]
	25	9.82 ± 0.46[a]	1.54 ± 0.05[a]	7.12 ± 0.39	1.88 ± 0.06[a]
	37.5	10.73 ± 0.75[a]	1.53 ± 0.18[a]	7.53 ± 0.42	1.69 ± 0.06[a]
	50	10.98 ± 0.44[a]	1.44 ± 0.04[a]	cytotox	cytotox

[a] $P < 0.05$ as compared to adequate vehicle (ethanol) control.

Table 6
Frequencies of micronuceated cytokinesis-blocked lymphocytes (MNCB) and nuclear division index (NDI) values in human lymphocytes treated with *B. officinalis, G. officinalis, V. hirundinaria* and *V. luteum* extracts.

Treatment (plant extracts)	Concent-ration (μg/mL)	Donor A		Donor D		Donor C	
		MNCB (‰ ± S.E.M.)	NDI (mean ± S.E.M.)	MNCB (‰ ± S.E.M.)	NDI (mean ± S.E.M.)	MNCB (‰ ± S.E.M.)	NDI (mean ± S.E.M.)
Blank		4.0 ± 2.0	1.51 ± 0.03	3.0 ± 1.7	1.38 ± 0.03	4.0 ± 2.0	1.49 ± 0.03
Ethanol	7 μL/mL	4.0 ± 2.0	1.49 ± 0.03	3.0 ± 1.7	1.35 ± 0.03	4.0 ± 2.0	1.51 ± 0.03
Doxorubicin	0.02	51.0 ± 7.0	1.34 ± 0.02	25.0 ± 4.9	1.13 ± 0.02	34.0 ± 5.7	1.55 ± 0.03
B. officinalis	50	2.0 ± 1.4	1.33 ± 0.02*	3.0 ± 1.7	1.31 ± 0.03		
	100	3.0 ± 1.7	1.64 ± 0.03*	3.0 ± 1.7	1.33 ± 0.03		
	150	3.0 ± 1.7	1.68 ± 0.03*	3.0 ± 1.7	1.23 ± 0.02*		
	200	4.0 ± 2.0	1.65 ± 0.03*	3.0 ± 1.7	1.20 ± 0.02*		
G. officinalis	12.5	7.0 ± 2.6	1.91 ± 0.04*	8.0 ± 2.8	1.34 ± 0.03		
	25	23.0 ± 4.7*	1.75 ± 0.04*	7.0 ± 2.6	1.41 ± 0.03		
	37.5	14.0 ± 3.7*	1.48 ± 0.03	11.0 ± 3.3*	1.29 ± 0.03*		
	50	17.0 ± 4.1*	1.59 ± 0.03*	8.0 ± 3.2	1.28 ± 0.03*		
V. hirundinaria	1.56					4.0 ± 2.0	1.43 ± 0.03*
	3.125					6.0 ± 2.4	1.39 ± 0.03*
	6.25					5.0 ± 1.0	1.21 ± 0.02*
	12.5					8.0 ± 2.8	1.09 ± 0.02*
V. luteum	1.56					2.0 ± 1.4	1.38 ± 0.03*
	3.125					5.0 ± 2.2	1.31 ± 0.03*
	6.25					8.0 ± 2.8	1.35 ± 0.03*
	12.5					4.0 ± 2.0	1.17 ± 0.02*

*$P < 0.05$ as compared to vehicle (ethanol) control.

observed in lymphocytes of donor D, however, this increase was statistically significant only after exposure to 37.5 μg/mL concentration. Interestingly that the same doses *G. officinalis* extract did not induced any significant increase of chromosome aberrations in human lymphocytes (Table 4). This could suggest that *G. officinalis* extract may have aneugenic properties. Consequently, this extract is a good candidate for further genotoxicity testing using mammalian *in vivo* tests.

NDI is a marker of cell proliferation in cultures and is considered to be a measure of general cytotoxicity (Eastmond and Turcker, 1989; Kirsch-Volders and Fenech, 2001; Fenech et al., 2003; Fenech, 2007). The decrease in NDI values may be attributed to several factors, both of general toxicity and DNA-damaging origin. Significant changes of nuclear division index (NDI) values were determined in treated cultures, indicating that all extracts were bioavailable for the cells. However, only *V. hirundinaria* and *V. luteum* extracts induced dose-dependent decrease of NDI values. Dose-response relationships were linear for both extracts and might be described by equations $y = 1.49–0.03x$ ($r = -0.98$, $P = 0.004$) for *V. hirundinaria* and $y = 1.45–0.02x$ ($r = -0.90$, $P = 0.037$) for *V. luteum,* where y is the frequency of MNCB (‰), x is the concentration of extract (μg/mL).

3.2.4. Comet assay

In the present study we used the alkaline modification of the comet assay which detects a mixture of DNA lesions including single- and double-strand breaks and alkali-labile sites (apurinic/apyrimidinic sites) (Collins et al., 2008). If such DNA damage is induced, broken DNA could migrate out of the nucleoid and form 'comet' with a distinct head, comprising intact DNA, and a tail, consisting of damaged or broken pieces of DNA. Thus, two measures of DNA migration are commonly used: comet tail length and percentage of the DNA in the comet tail (%TDNA), the latter is linearly related to DNA break frequency and thus is considered as the most reliable parameter (Collins et al., 2008). For this reason, %TDNA was a parameter of choice in our study.

We performed comet assay on isolated peripheral blood lymphocytes of two female donors, A and B. We used hydrogen peroxide (H_2O_2, 20 μM) as a positive control, which induced significant increase in %TDNA in lymphocytes of both donors (26.97 ± 10.71 and 30.64 ± 21.55 for donors A and B, respectively). The results of the comet assay revealed different genotoxic potential of the plant extracts (Fig. 1). *V. hirundinaria* and *V. luteum* extracts induced statistically significant increase in %TDNA in comparison with the control in lymphocytes of both donors, however, no dose-dependent relationship was determined. *B. officinalis* and *G. officinalis* extracts induced DNA damage in a dose-dependent manner. The dose-response relationships for *B. officinalis* most adequately may be described by the linear equations: $y = 0.89 + 0.11x$ ($r = 0.97$, $P = 0.006$, donor A) and $y = 0.3 + 0.03x$ ($r = 0.90$, $P = 0.015$, donor B), where y is the %TDNA and x is the concentration of the plant extract (μg/mL). The established dose-response relationships for *G. officinalis* were $y = 2.40 + 0.03x$ ($r = 0.91$, $P = 0.033$, donor A) and $y = 1.44 + 0.27x$ ($r = 0.95$, $P = 0.004$, donor B).

3.2.5. Plant phytochemicals and bioactivities

Iridoids were found as the main phytochemicals in the *Stachys* genus plants (Tundis et al., 2014; Háznagy-Radnai et al., 2006a,b; Vundać et al., 2007, 2019). Luca et al. (2019) recently reported that methanolic extract and its fractions of *Verbascum ovalifolium* Donn ex Sims containing strong antioxidants (mainly catalpol-type iridoid diglycosides) showed a significant *in vitro* antioxidant capacity, antiproliferative activity in malignant melanoma SK-MEL-2 cells and no important cytotoxicity and genotoxicity in V79 cells. Major iridoid glycoside of *Harpagophytum procumbens*, harpagoside significantly reduced the mutagenicity of 1-nitropyrene in the assay using the *in vitro* cytokinesis-block micronucleus in cultured human lymphocytes (Manon et al., 2015). In general, iridoid glycosides possess various bioactivities; for instance, they might modulate T cell-related pathologies (Dimitrova et al., 2018) and protect uric acid nephropathy rats (Hou et al., 2014). Regarding phenolic acids, which were also among the main

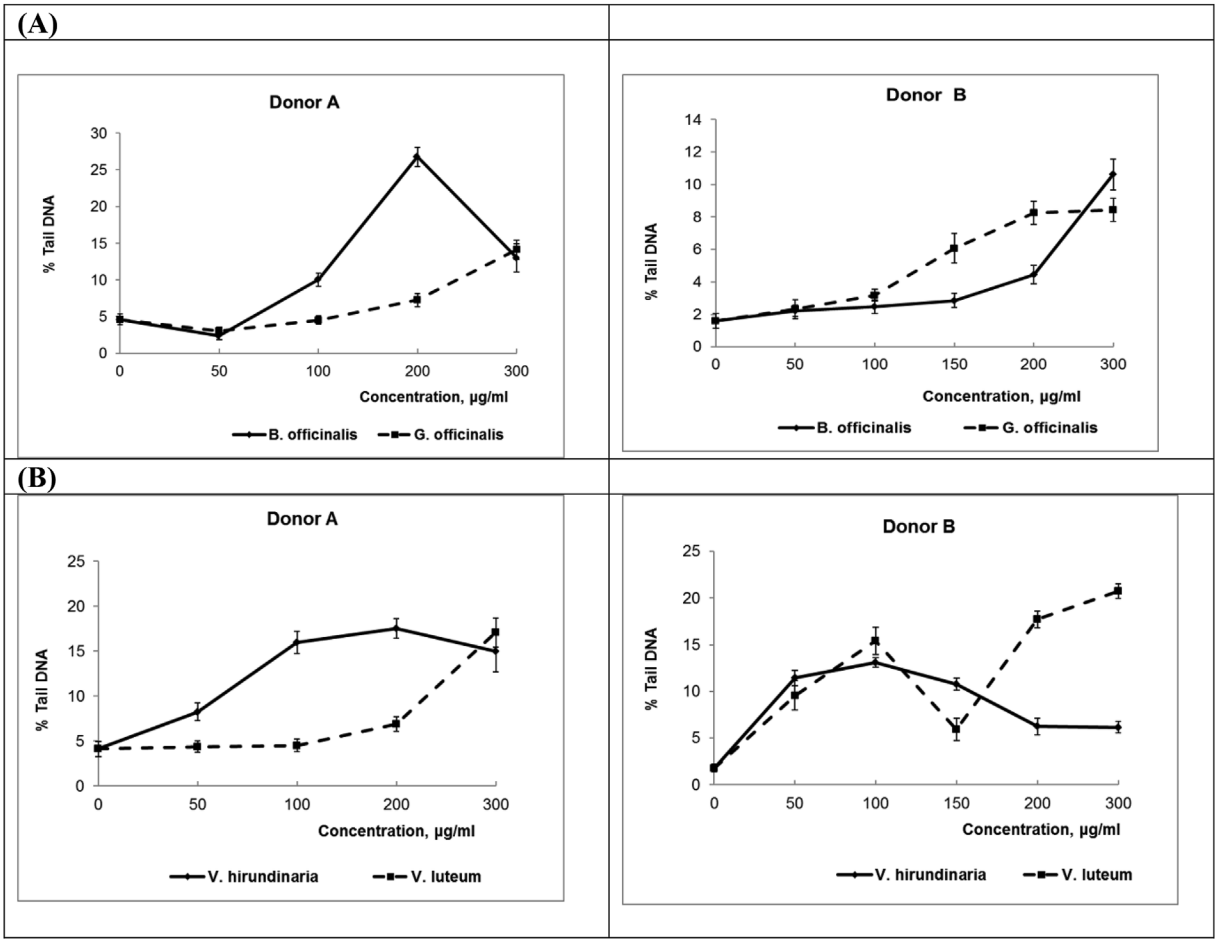

Fig. 1. The results of the genotoxicity testing of *B. officinalis, G.officinalis* **(A)**, *V. hirundinaria* and *V. luteum* **(B)** plant extracts by the comet assay expressed as mean percentages of DNA in the comet tail (%TDNA) ± S.E.M.

phytochemicals in acetone and methanol extracts of woody betony (Šliumpaitė et al., 2013a), many of them were reported as possessing antimutagenic properties. For instance, coffee extracts containing chlorogenic acid derivatives exhibited strong antimutagenic activity against the oxidizing factor *tert*-butyl hydroperoxide in the Ames test (Priftis et al., 2018).

In the *in vivo* studies with rabbits, verbascoside, which is one of the most active phenylporpanoids reported in *G. officinalis* (Šliumpaitė et al., 2013b), did not reveal any mutagenic effects on chromosomes using the cytogenetic tests, namely chromosome abnormalities in terms of chromosome and chromatid breaks and sister chromatid exchange (Perucatti et al., 2018). Moreover, *G. officinalis* reduced the mutagenic action of cyclophosphamide in peripheral blood erythrocytes from outbred white mice at a dose of 200 mg/kg (Durnova and Kurchatova, 2015) and exhibited high anti-inflammatory activity.

Chlorogenic acid and isoquercitrin were major phenolics in *V. lutea* (Šliumpaitė et al., 2013c). The latter flavonoid is a common component of a normal human diet. Valentova et al. (2016) reported negligible mutagenicity for isoquercitrin using the Ames test in *Salmonella typhimurium* His(−) strains TA100, TA98 and TA102. However, the compound showed protective activity against H_2O_2- and *tert*-butyl hydroperoxide-induced oxidative damage to the TA102 strain. Consequently, existing information on the phytochemical composition of the studied plants, although still remaining rather limited (except for *B. officinalis*), as well as the reported bioactivities of the main identified constituents supports our results about the absence (or in some case negligible) genotoxicity/mutagenicity of extracts prepared from these botanicals.

4. Conclusion

Extracts isolated from *Betonica officinalis, Gratiola officinalis, Vincetoxicum luteum* and *Vincetoxicum hirundinaria* demonstrated strong antioxidant potential and were not mutagenic in *S. typhimurium* strains TA98 and TA100 with and without metabolic activation and did not induce chromosome aberrations in human lymphocytes *in vitro*. Extract from *Gratiola officinalis* induced micronuclei but not chromosome aberration in human lymphocytes *in vitro*, indicating possible aneugenic effect. This extract is the first candidate for further genotoxicity testing using mammalian tests *in vivo*. All investigated medicinal plant extracts induced DNA damage evaluated by the comet assay, and extracts from *B. officinalis* and *V. luteum* induced slight increase in SCE values. The determined variation in response might be due to the plant extract tested and donor susceptibility. The results of this study may serve as important supplementary data in further valorization of the selected plants for various applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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