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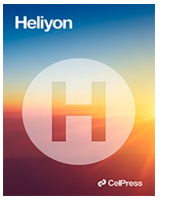

Research article

Assessment of the biological activity of *Marrubium friwaldskyanum* Boiss. (*Lamiaceae*)

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A B S T R A C T

Present scientific evidences about the biological activity and potential medical application of extracts derived from *Marrubium friwaldskyanum* Boiss. are limited. Therefore, our study was undertaken to define several main characteristics in this regard – *in vitro* cytotoxicity and antitumor properties, antibacterial activity and immunomodulatory potential. Extracts were obtained from different aerial parts of *Marrubium friwaldskyanum* – stems, leaves and flowers. The *in vitro* cytotoxicity and antitumor activity of the samples were evaluated by tetrazolium salt reduction tests and Neutral red uptake assays using four human cell lines (a normal fibroblastic and three adenocarcinoma cell lines/A549, HeLa, HT-29/) and by experiments with HT-29 tumor spheroids. Antibacterial activity toward Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus cereus*) species was assessed based on estimation of minimal inhibitory and minimal bactericidal concentrations as well as longitudinal studies on bacterial viability. *Ex vivo* assays with normal leukocytes were performed to define potential immunomodulatory activity of the extracts. Our results demonstrated selective antitumor activity of the extracts directed against colon adenocarcinoma HT-29 cells and cervical adenocarcinoma HeLa cell line. Metabolic activity of A549 lung adenocarcinoma cells was affected only by the sample derived from flowers. *M. friwaldskyanum* leaf and flower extracts showed the highest activity, which included reduction of HT-29 tumor spheroid growth and viability. The studied samples exhibited antibacterial activity against both bacterial species tested. Treatment with *M. friwaldskyanum* extracts affected specific leukocyte populations (HLA^+, $CD19^+$, $CD11b^+$, $CD25^+$ cells). These results demonstrate for the first time complex biological effects of extracts derived from *M. friwaldskyanum* and their potential to serve as a source of valuable compounds for the pharmaceutical industry.

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1. Introduction

Certain species of the genus *Marrubium* (Lamiaceae) are well known for their diverse biological activity and benefits for human health. They are used in traditional medicine as analgesics, antipyretic, diuretic or sedative agents for treatment of various diseases (infections, asthma, cancer, liver diseases, diabetes, inflammatory conditions, hypertension, gastrointestinal problems etc.), [1–4]. Among them are *M. vulgare*, *M. parviflorum*, *M. globosum*, *M. peregrinum, M. deserti* and others [1,4–7]. Essential oils and extracts obtained from these herbs have shown rich composition of natural compounds including sesquiterpenes, phenolic acids, flavonoids, diterpenes and diterpenoids, iridoids and their glycosides, coumarins, and sterols [7–12] – content that supports the pharmacological potential of the respective plants [13]. However, there are a number of *Marrubium* species with limited distribution that have not been intensively studied. A typical example is *Marrubium friwaldskyanum* Boiss. – an endemic organism growing in two main regions in Bulgaria: the west and middle parts of Rhodopes Mountain and the Thracian Plain [14], at an altitude range of 200–1400 m [15]. This perennial herbaceous plant shows morphological similarities with *M. vulgare* [16,17], which is the most studied representative of the genus *Marrubium* [1], commonly used in folk medicine in different countries and a popular herbal supplement in USA (holding the second position in top 10 ranking of herbs sold by the US mainstream multi-outlet retail channels) [18]. Embryological studies have defined high reproductive capacity of *M. friwaldskyanum* and it has been suggested that cultivation of the plant on rich soils might lead to significant yields that are comparable to the production obtained by cultivation of *M. vulgare* [16]. Further experiments are needed to support this hypothesis, but it is clear that *M. friwaldskyanum* could be used as a source of biologically active compounds. Recent phytochemical investigations proved the presence of a number of important natural compounds in essential oils and extracts derived from *M. friwaldskyanum* including sesquiterpenes, flavonoids, triterpenoids and phenolic acids [16,19]. These findings point to a biomedical potential of the plant that has not been well characterized to date. Studies by Kozyra et al. indicated cytotoxic activity of a non-hydrolyzed methanolic fraction of *M. friwaldskyanum* extract against melanoma cell line [20]. Other reports on *M. friwaldskyanum* biological activities have not been reported.

A significant number of specific compounds are present in *M. friwaldskyanum* extracts [16,19]. In addition, we found unique tissue-specific compounds for *M. friwaldskyanum* flower and leaf extracts [19]. They could exhibit diverse biological activities like antitumor, anti-inflammatory, immunomodulatory, antibacterial, antiparasitic and other beneficial properties. Although total extracts represent a complex mixture of bioactive compounds analyses of their activity reveal different aspects of the biomedical potential of particular plant. Part of such properties could be based on synergistic effects of different components of the extract that cannot be defined in purified samples. To date such evaluations of *M. friwaldskyanum* extracts have not been reported, which motivates the scope of the present study. Our metabolome analyses of samples obtained from particular aerial parts of the herb (flowers, leaves, stems) showed rich composition with pharmacological potential [19]. Therefore, this is a continuation of our previous work aimed to evaluate the biological activity of *M. friwalskyanum* leaf, flower and stem extracts in three main aspects – antitumor activity, antibacterial ability and immunomodulatory potential. Two classical *in vitro* cytotoxicity assays were used to analyze the inhibitory effects of the plant extracts against normal cells and different tumor cell types – a tetrazolium salt test measuring metabolic activity of viable cells and Neutral red uptake assay that determine cellular viability based on lysosomal activity. Thus, we were able to determine selective antitumor effects or general cytotoxicity with impact on specific cellular structures – mitochondria and lysosomes. The antitumor activity analyses proceeded with determination of the inhibitory potential on the multicellular level using 3D tumor spheroids. Following treatment with *M. friwalskyanum* extracts the spheroid size and viability were determined as main indicators of tumor cell aggregate suppression. Standard microbiological analyses were performed to define the minimum inhibitory concentration and the minimum bactericidal concentration of the samples toward Gram-negative and Gram-positive bacterial species. In addition, the potential longitudinal inhibitory effects were studied in order to better characterize the antibacterial potential of *M. friwalskyanum* extracts. Finally, effects on main immunological markers were investigated performing *ex vivo* treatment of peripheral blood leukocytes with the plant samples with determined antitumor effects. These experiments indicated the potential influence of *M. friwalskyanum* extracts on the immune system activity that could contribute to the organism defense against infections but also against tumor cells. Overall, the present report provides multilateral assessment of the biological properties of an endemic *Marrubium* species with good reproductive potential growing in Bulgaria.

2. Materials and methods

2.1. Plant material and preparation of extracts

The endemic species *M. friwaldskyanum* Boiss. is included in the Red Book of Bulgaria [21] and according to Bulgarian legislation, its collection is under a special regime of regulation. In order to ensure a sufficient amount of plant material needed for the planned phytochemical and biological analyses, flowers, leaves and stems were collected during the summer of year 2020 and 2021 from natural *M. friwaldskyanum* populations located near Fortress Cepina, Western Rhodopes Mountain (42°09′N; 24°12′E/1136 m). Plants were identified by Prof. Plamen Stoyanov and example specimens were stored at the Herbarium of the Agricultural University (SOA), Plovdiv, Bulgaria under number 063316. The collected herb material was dried in a dark place at ambient temperature. The dried plant mass was mechanically grinded and powder containing particles with size lower than 400 μm was obtained. The samples were stored in tightly sealed vials at 16–18 °C in a dark room. The extraction of *M. friwaldskyanum* powder samples obtained from flowers, leaves and stems (10 g each) was performed as previously described [19]. GC–MS, UPLC-MS/MS and ICP-MS data related to the phytochemical content of *M. friwaldskyanum* extracts were reported [19]. The resulting polar extracts were filtered using Whatman No.1 paper filters (Merck KGaA, Darmstadt, Germany). After that, vacuum evaporation of methanol was performed at 37 °C using Savant apparatus

(SAVANT Instruments Inc., Farmingdale, NY, USA). All plant extracts were stored at 4 °C in a dark environment. Prior the evaluations of biological activity, 100 mg flower, leaf and stem extract were dissolved in 10 mL Dulbecco's phosphate buffered saline (DPBS, Merck KGaA, Darmstadt, Germany) and used immediately.

2.2. Cell lines and culture maintenance

To evaluate the cytotoxicity and possible antitumor properties of *M. friwaldskyanum* extracts *in vitro* assays were performed using four human cell lines: A549 (lung adenocarcinoma, ATCC CCL-185™), HeLa (cervical adenocarcinoma, ATCC CCL-2™), HT-29 (colorectal adenocarcinoma, ATCC HTB-38), and HFFC (fetal foreskin fibroblasts provided by CLS Cell Lines Service GmbH, Eppelheim, Germany). These cell types were selected due to the general worldwide prevalence of lung, colorectal and cervical tumors. They are among the 10 most common and fatal cancer diseases as indicated by the International agency for research on cancer (https://gco.iarc.fr/en). HFFC cells served as a control of normal cells in the assays for antitumor activity. All cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 μg/mL streptomycin,100 IU penicillin) (all purchased from Merck KGaA, Darmstadt, Germany), denoted as complete DMEM or supplemented DMEM. HFFC cells were grown in DMEM with higher FBS content (15 %) and the same concentration of antibiotics. Before performing the assays, all cell types were propagated in 75 cm^2 culture flasks (TPP, Trasadingen, Switzerland) and trypsinized at 80 % confluency. The cells were kept in incubator maintaining appropriate culture conditions (37 °C, high humidity, gas phase composed of a mixture of 5 % CO_2/95 % atmospheric air).

2.3. Assessment of antitumor activity in monolayer cell cultures

Two classical *in vitro* cytotoxicity assays were performed to analyze the antitumor activity of *M. friwaldskyanum* extracts – a test that measures cellular metabolic activity based on the capability of functional cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide (MTT) (MTT assay) [22], and Neutral red (NR) uptake assay (NRU assay) that determines cell viability through assessment of NR quantity accumulated in the lysosomes of live cells [23]. For these experiments, cell suspensions with 1×10^5 cells/mL concentration were prepared from all four cell lines and seeded on 96-well plates (TPP, Trasadingen, Switzerland) (100 μL/well). The established cultures were grown under standard conditions for 24 h. After that, *M. friwaldskyanum* extracts in different concentrations (10, 100, 250 and 500 μg/mL) were added to the culture medium for a test-period of 24 and 72 h. To achieve this, 1 mg/mL working solutions were prepared from the extract stock solutions by dilution in complete DMEM medium and then, a particular amount of test-sample was pipetted on the culture plates to obtain the appropriate test-concentration per well. 10 mg/mL extract stock solutions were prepared using DPBS. Thus, negative cellular effects induced by the solvent were avoided. The final volume of all assayed samples was 200 μL/well. All tests included a positive control - mitomycin C (MMC), analyzed in the same test-concentrations as the extract samples. Also, a control with untreated cells cultured in appropriate growth medium for the same test-period was assayed in all experiments.

After the end of the two test-periods (24h and 72h), the effects of *M. friwaldskyanum* extracts on cellular metabolic activity and viability were analyzed. For MTT assays: MTT solution (Merck KGaA, Darmstadt, Germany) in a final concentration of 0.5 mg/mL was added to the growth medium of the cells. The cultures were incubated for 2 h at 37 °C, 5 % CO_2 and high humidity in dark environment. The growth medium was then removed which was followed by washing with DPBS. The formazan accumulated in the cells after incubation with MTT-containing medium was solubilized using 100 μL/well dimethyl sulfoxide (DMSO, Merck KGaA, Darmstadt, Germany). The plates were then incubated at ambient temperature on a shaker for 15 min. Absorbance was measured at 570 nm using a SpectraMax i3x spectrophotometer (Molecular devices, San Jose, CA, USA).

For the NRU assays NR solution (Merck KGaA, Darmstadt, Germany) was added to the cell culture medium to reach 0.005 mg/mL final concentration. After 2-h incubation at 37 °C, 5 % CO_2 and high humidity the cells were washed with DPBS. 100 μL/well aqueous solution of ethanol and glacial acetic acid (50 %:1 %) was then added to the cells in order to extract the accumulated NR stain. The culture vessels were incubated for 15 min at room temperature with continuous mild shaking. After that, absorbance at 540 nm was analyzed using a SpectraMax i3x spectrophotometer (Molecular devices, San Jose, CA, USA). All samples were plated in triplicates for MTT and NRU *in vitro* cytotoxicity assays. The obtained data was used to determine percent inhibition of cell viability and/or metabolic activity based on absorbance results for treated cells and cells cultured under standard conditions without the addition of extract. The concentration of the test-sample that inhibited the viability/metabolic activity of 50 % of the cells (IC_{50}) was calculated for the 72-h test-period.

2.4. In vitro assays with tumor spheroids

HT-29 cells were used to obtain tumor spheroids on 96-well round-bottom Corning® spheroid microplates (Corning Inc., Glendale, AZ, USA) with ultra-low attachment surface coating of the wells. For this aim, 5000 cells/well in 200 μL volume were seeded and after 24h 3D spheroid formation was verified. Then, *M. friwaldskyanum* extracts were added in a final concentration of 250 μg/mL and the spheroids were cultured for 96 h. Spheroids grown for the same test period in complete DMEM were analyzed during the assays providing untreated control. Measurements of spheroids diameter after 24 and 96 h of culture after addition of test-sample were performed using inverted microscope Inverso (Medline Scientific, Chalgrove, Oxon, UK) equipped with high-resolution Si-3000 digital camera and specialized software (Medline Scientific, Chalgrove, Oxon, UK). Ten spheroids were measured for each sample.

The viability of HT-29 cells comprising spheroids was evaluated by staining with 5-carboxyfluorescein diacetate, acetoxymethyl

ester (5-CFDA, AM) (Invitrogen, Molecular probes™, Eugene, OR, USA). This reagent is capable to pass the cell membrane and penetrate into the cytoplasm where active esterases convert it to a fluorescent form - 5-carboxyfluorescein. The fluorescent staining is characteristic for viable cells with unaffected esterase activity and intact membrane that retain the dye intracytoplasmically. 5-CFDA, AM in a final concentration of 2.7 μg/mL was added to spheroid cultures treated for 96h with *M. friwaldskyanum* extracts. The spheroids were incubated at 37 °C in the dark for 30 min. Then, the medium containing 5-CFDA, AM was replaced with DPBS and the staining of 3D multicellular structure was analyzed on a fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

2.5. Analyses of antibacterial properties

The antibacterial activity of *M. frivaldskyanum* samples was estimated by experiments with Gram-negative and Gram-positive species – *Escherichia coli* (ATCC 25922) and *Bacillus cereus* (ATCC 11778), respectively. The bacteria were grown at 37 °C in Mueller Hinton agar or broth medium (Merck KGaA, Germany). The minimum inhibitory concentration (MIC) of *Marrubium* extracts against *B. cereus* and *E. coli* was defined using the plate microdilution method described by Moyo & Mukanganyama [24]. For these experiments, two-fold serial dilutions were prepared from all samples, as well as from an antibiotic control ceftriaxone (MIP Pharma GmbH, Blieskastel-Niederwurzbach, Germany) starting from a concentration of 2 mg/mL 100 μL of the sample with 2 mg/mL concentration and the diluted samples were pipetted into a 96-well plate. Then, 100 μL of bacterial suspension was added to all samples to a final concentration of 1×10^6 cfu/mL. As a result, the highest concentration of test-sample was 1 mg/mL. In all assays of this type, the following controls were used: 200 μL broth containing 100 μL M. *friwaldskyanum* extract and 100 μL growth medium; 200 μL broth that contained 100 μL culture medium and 100 μL bacteria. The culture dishes were incubated for 24 h at 37 °C, after which 5 mg/mL MTT solution (20 μL/well) was added to each well. In the presence of metabolically active cells in the studied sample, MTT (yellow tetrazolium salt) undergoes reduction that yields a formazan product with purple color. The plates were incubated for 1 h at 37 °C, after which absorbance was measured at 570 nm using a SpectraMax i3x spectrophotometer (Molecular devices, San Jose, CA, USA).

To define the minimum bactericidal concentration (MBC), 50 μL of the samples, determined as the MIC, were inoculated into two separate Petri dishes containing antibiotic-free nutrient agar. In addition, one or two samples corresponding to concentrations higher than the MIC and one sample with a concentration lower than the MIC were also tested. The established cultures were incubated for 24 h at 37 °C. The concentration of the test sample at which growth of bacterial colonies was not detected was defined as MBC.

Additional experiments were performed to evaluate the effects of *M. friwaldskyanum* extracts on bacterial viability over time. 3 mL cultures with concentration 1×10^6 cfu/mL were prepared and maintained for 36 h at 37 °C. For each bacterial species (*E. coli* or *B. cereus*) four samples were established: untreated control in unmodified broth, a culture treated with 400 μg/mL M. *friwaldskyanum* flower extract, bacteria grown in medium containing 400 μg/mL M. *friwaldskyanum* leaf extract, and culture maintained in medium supplemented with 400 μg/mL M. *friwaldskyanum* stem extract. All samples were examined in triplicates. Throughout this experiment, at 2, 12 and 24 h culture period, and at the end of the assay (36 h) 100 μL samples from the bacterial suspensions were obtained. To evaluate bacterial viability over time the samples were set on a 96-well culture plate (Costar, Corning Inc., New York, NY, USA). Then, MTT solution (Merck KGaA, Darmstadt, Germany) in 5 mg/mL concentration was added to each sample well and diluted tenfold in the broth. The plates were incubated for 1 h at 37 C. During this incubation period viable bacteria in the samples reduce the yellow-colored MTT to a purple-colored formazan product [23]. and then absorbance at 570 nm was determined by measurement on a spectrophotometer SpectraMax i3x (Molecular devices, San Jose, CA, USA).

2.6. Experiments with peripheral blood leukocytes

Venous blood from 4 healthy volunteers (2 men and 2 women, 28–39 age) was obtained. The samples were collected in BD Vacutainer® K2EDTA tubes (Becton, Dickinson and Company (BD), NJ, USA) and standard hematological parameters were analyzed. Consequently, reference range values were determined for all examined parameters. All obtained samples were centrifuged at 1000×*g* for 15 min. After that the plasma was discarded. The cell pellets were gently resuspended in 0.84 % NH_4Cl buffer and incubated at room temperature for 8–10 min in order to lyse erythrocytes in the samples. This was followed by washing with DPBS. The samples were washed twice. The resulting leukocyte fractions were pooled and centrifuged for 10 min at 1000×*g*. Then the cells were resuspended in supplemented DMEM and 1×10^6 cells/mL were seeded on a 12-well culture plate (TPP, Trasadingen, Switzerland). *M. friwaldskyanum* extracts in a final concentration of 200 μg/mL were added to the cultures which were grown under standard conditions for 24 h. Control leukocytes cultured in supplemented DMEM without plant extract was also set. All test-samples were assayed in triplicates. The experiment was approved by the Local Ethical Committee at "Paisii Hilendarski" University of Plovdiv, Bulgaria (protocol No. 5 from June 10, 2020) and performed in accordance to the Declaration of Helsinki Blood sampling was implemented in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki) approved for experiments involving humans. All volunteers were informed about the purpose of the experiment and signed a written informed consent before blood sampling and initiation of the experimental procedure.

2.7. Immunophenotyping

The leukocyte cultures were kept at 37 °C in an incubator with humidified atmosphere containing 5 % CO_2. After the 24-h test-period, cells from the control and treated cultures were harvested, centrifuged for 10 min at 1000×*g* and resuspended in buffer for flow cytometry (DPBS, containing 5 % FBS and 0.05 % NaN_3). The cells were incubated with fluorochrome-labeled antibodies specific for the markers CD3, CD4, CD8, CD19, CD25, CD11b and HLA-DQ/DR/DP (BD Pharmingen™, BD Biosciences, USA), and incubated for

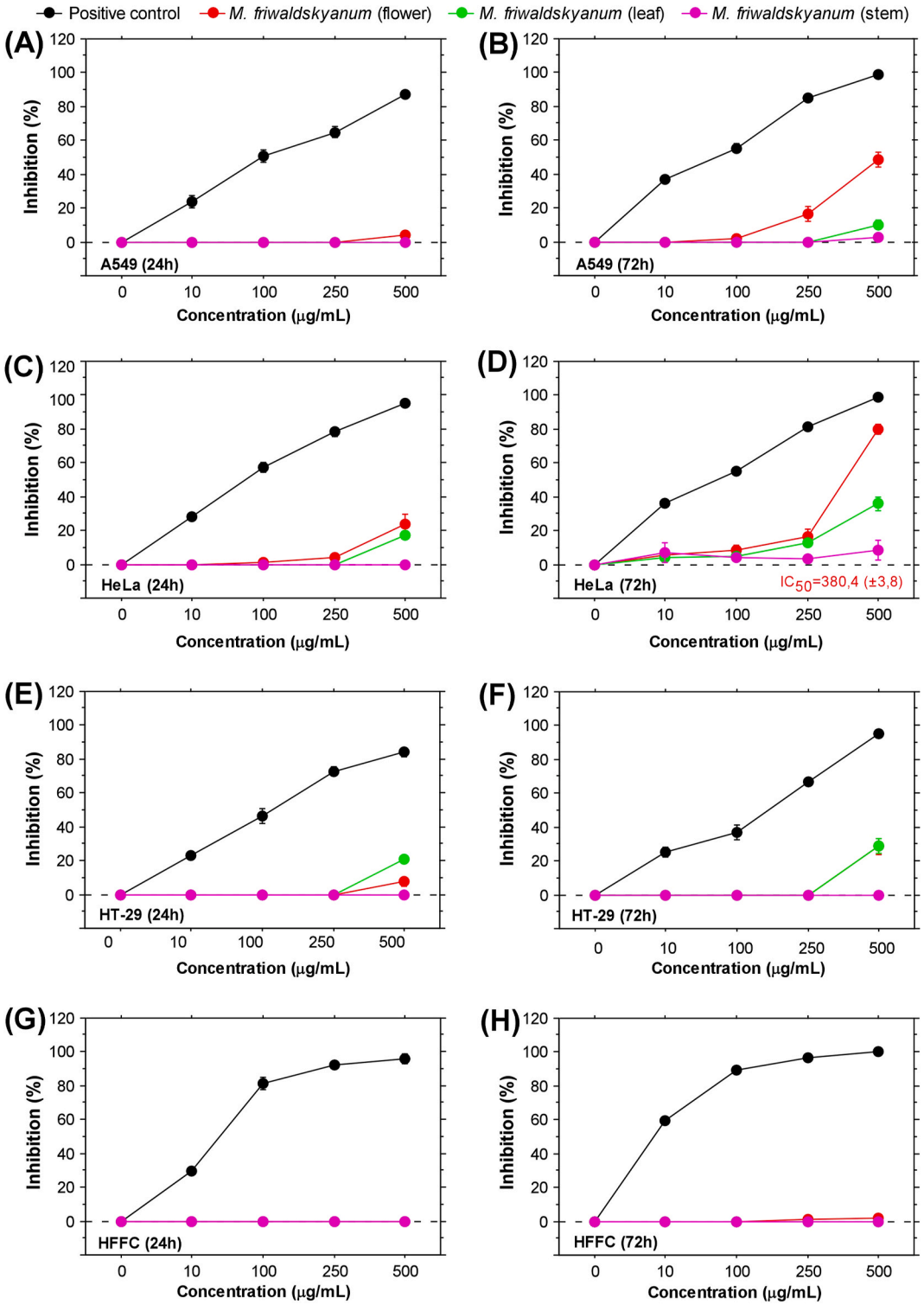

Fig. 1. Inhibition of cellular metabolic activity after treatment with *M. friwaldskyanum* extracts. The graphs show MTT assay data for A549 cells (**A**, **B**), HeLa cells (**C**, **D**), HT-29 cells (**E**, **F**) and normal HFFC fibroblasts (**G**, **H**). Positive control = Mitomycin C (MMC). The results represent ± standard error of the mean (±SEM). All samples were analyzed in triplicates. Statistically significant differences between *M. friwaldskyanum* extracts and the positive control were analyzed. Significant p values ($p < 0.05$) were determined for all samples and all test-concentrations (10–500 µg/mL).

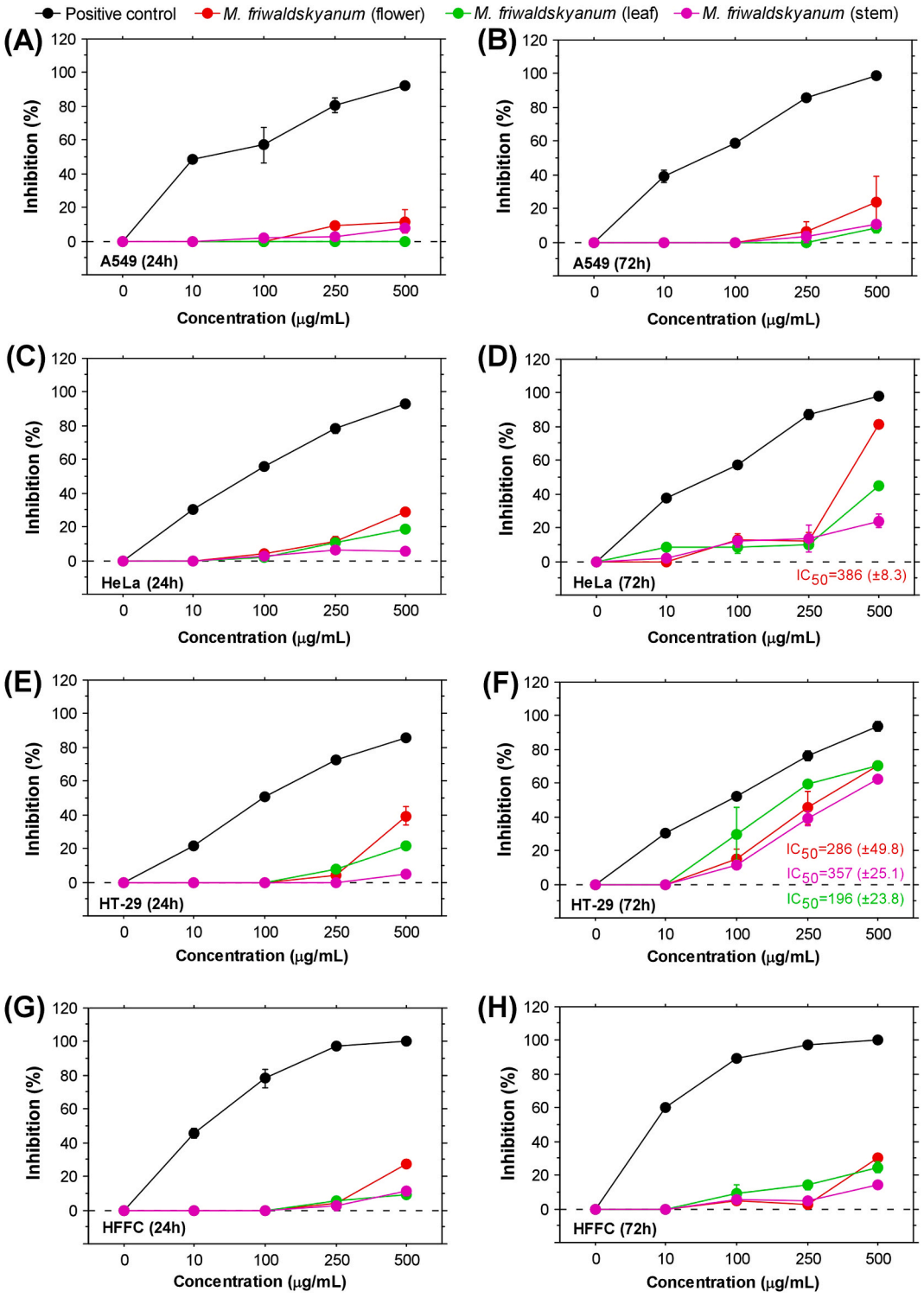

Fig. 2. Neutral red uptake assay results obtained after treatment for 24 and 72 h with *M. friwaldskyanum* flower, leaf and stem extracts. The charts show: **A**, **B** - A549 cells, **C**, **D** - HeLa cells, **E, F** - HT-29 cells and **G, H** - HFFC fibroblasts. Positive control = Mitomycin C (MMC). The results represent ± SEM. All samples were analyzed in triplicates. Statistically significant differences between *M. friwaldskyanum* extracts and the control were analyzed. Significant p values ($p < 0.05$) were determined for all samples and all test-concentrations (10–500 μg/mL).

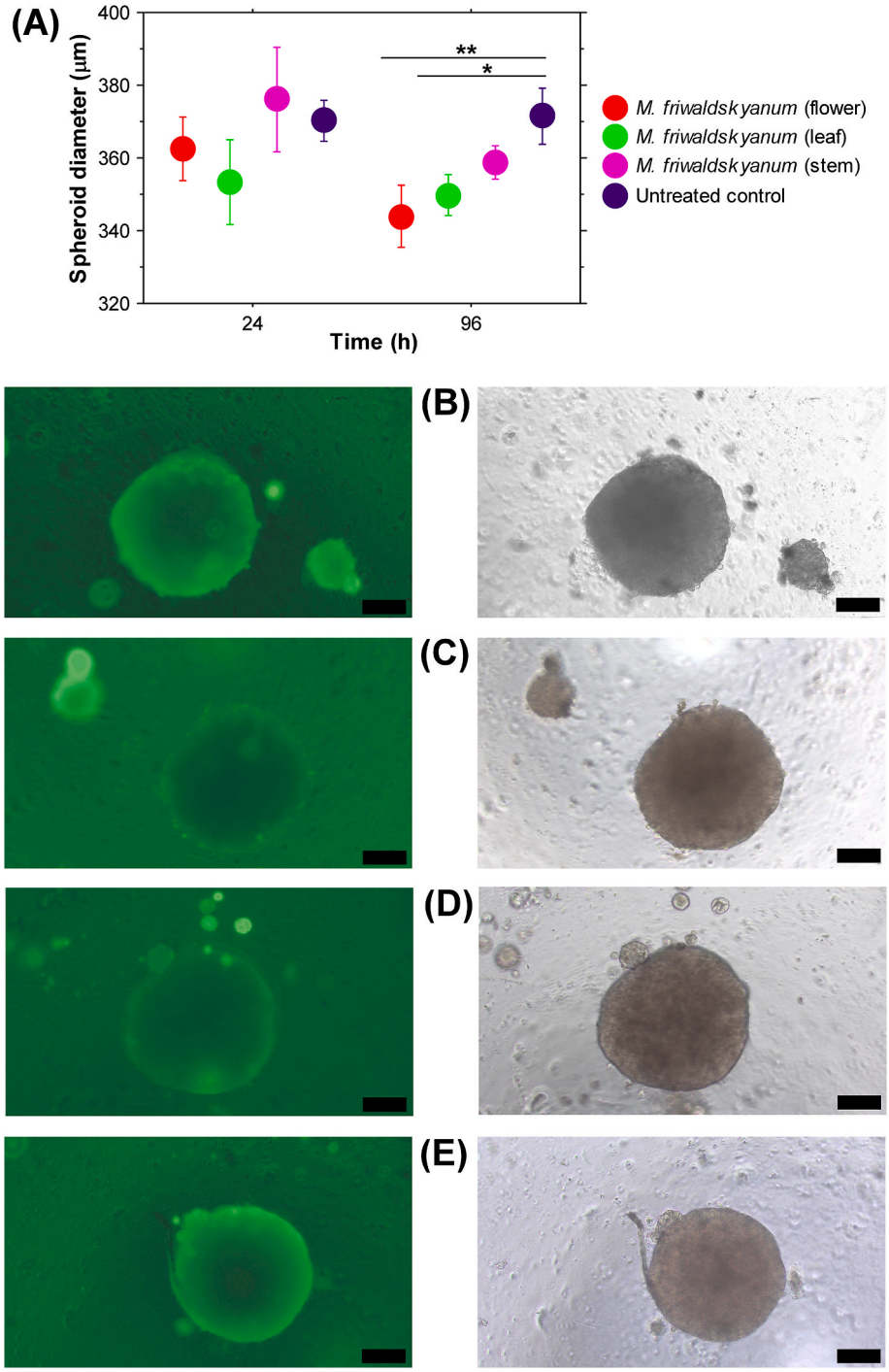

Fig. 3. Effects of *M. friwaldskyanum* extracts on tumor spheroids. (**A**) Measurements of spheroid diameter after 24 and 96 h treatment with *M. friwaldskyanum* extracts. 10 spheroids per sample were analyzed. The results are present as ±SEM. Untreated spheroids (control) – fluorescent and bright field image (**B**); HT-29 spheroids treated with *M. friwaldskyanum* extracts for 96 h (**C-E**); fluorescent microscopy and bright field image of sample treated with flower extract (**C**); fluorescent and bright field image of spheroid treated with leaf extract (**D**); fluorescent microscopy and bright field image of sample treated with stem extract (**E**). The black bar correspond to 100 μm *, $p < 0.05$; **, $p < 0.01$.

20 min in dark environment. After that the cells were washed twice and analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter Inc., Life Sciences, Indianapolis, IN, USA). Different leukocyte populations were compared between the control and extract-treated samples. The results were shown as mean percentages ± SD (n = 3).

2.8. Statistical analyses

Student's t-test (StatView software, version 5.0) (SAS Institute Inc., Carry, NC, USA) was used to determine statistical significance of the obtained results. Analysis of variance (ANOVA) was applied to reveal differences between the controls and the test-groups. Values of p that were lower than 0.05 were considered statistically significant.

3. Results

3.1. Antitumor activity of M. friwaldskyanum

The ability of *M. friwaldskyanum* extracts to inhibit the growth, activity and vitality of different tumor cell types was investigated *in vitro*. For this aim, MTT tests that estimate mitochondrial metabolic activity and NRU assays that determine cellular viability were performed. In addition, multicellular HT-29 tumor spheroids size and viability were analyzed following treatment with plant extracts.

3.1.1. Selective inhibition of adenocarcinoma adherent cultures

Responses of lung, cervical and colon adenocarcinoma cell lines (A549, HeLa and HT-29, respectively) against treatment for 24 and 72 h with different concentrations of *M. friwaldskyanum* extracts were analyzed. The results were compared with data obtained with normal fibroblastic finite line (HFFC) to determine eventual antitumor properties of the three analyzed samples – flower, leaf and stem extracts. Fig. 1 represents results from MTT assays. They indicate time- and concentration-dependent inhibition of cellular metabolic activity induced by treatment with *M. friwaldskyanum* extracts (Fig. 1A,B,C,D,E,F). Intriguingly, normal fibroblasts metabolic activity was not affected by the plant samples present in different concentrations in the cell culture medium (Fig. 1G and H), which proves the antitumor properties of *M. friwaldskyanum.* The three tumor cell lines showed different modes of inhibition induced by the flower and leaf extract (Fig. 1A,B,C,D,E,F). Their effects increased after longer culture period in the presence of the test-samples (72 h). The sample derived from *M. friwaldskyanum* flowers caused the highest inhibition of A549 and HeLa cells (Fig. 1B and D) while the leaf and the flower extract induced similar level of inhibition in HT-29 cultures (Fig. 1F). Concentrations of flower extract that inhibit 50 % of cellular metabolic activity (IC_{50}) for HeLa cells were determined and indicated on the respective graphs.

M. friwaldskyanum extracts induced stronger effects on the level of lysosome functionality and cellular vitality, which was determined by the NR uptake assays (Fig. 2). We observed time-dependent and concentration-dependent responses by all cell types. A459 (Fig. 2A and B) and HFFC cells (Fig. 2G and H) showed weak inhibition following treatment with 250 and 500 μg/mL extract. It is evident that NRU assays demonstrated inhibitory effects of the test-samples on normal fibroblasts. However, their level was markedly lower compared to the inhibition measured for HeLa (Fig. 2C and D) and HT-29 cells (Fig. 2E and F). These results point to selective antitumor properties directed to cervical and colon adenocarcinoma cells. Concordant with MTT assay data the flower and leaf extracts were most effective in reduction of cellular viability. In addition, the extract derived from *M. friwaldskyanum* stems demonstrated inhibitory activity after 72-h treatment that was highest against HT-29 cells. The colon adenocarcinoma cell line was the most sensitive to treatment with *M. friwaldskyanum* extracts. More than 50 % inhibition was detected after 72-h cultivation of HT-29 cells in medium containing flower, leaf and stem extracts (Fig. 2F). HeLa cells showed very similar IC_{50}values calculated based on reduction of cellular vitality and lysosome functionality – 380.4 μg/mL for MTT test (Fig. 1D) and 386 μg/mL for NRU assays (Fig. 2D). Generally, the results from MTT and NRU assays for HT-29 cells indicated predominant lysosome-specific mechanism of cytotoxicity of *M. friwaldskyanum* extracts.

3.1.2. Reduction of tumor spheroid growth and vitality

In order to perform more detailed characterization of the antitumor potential of *M. friwaldskyanum*, the *in vitro* cytotoxicity assays with adherent cell cultures were followed by experiments with HT-29 tumor spheroids. Consequently, these studies could provide information about the inhibitory potential of the extracts on the multicellular level. For these experiments spheroid microplates with ultra-low attachment surface coating were used. The manufacturer of these vessels has shown that they could be used for generation of HT-29 and A549 3D tumor spheroids. However, A549 cells were not included in our analyses because they showed the least sensitivity to *M. friwaldskyanum* extract treatment during the MTT and NR uptake assays compared to HT-29 and HeLa cells. We have tested the spheroid microplates with HeLa but the cells formed loose aggregates that were not structured as compact spheroids. Therefore, we excluded the HeLa cell line from the spheroid analyses. For the experiments, HT-29 spheroids were cultured in medium containing 250 μg/mL M. *friwaldskyanum* extract for 96 h. The test-concentration was selected based on the MTT and NRU data. We wanted to determine whether an extract concentration lower than the maximal that induce inhibition, but to a lower extent, could affect tumor spheroids. During the experiments tumor cell aggregates diameter was determined following 24-h and 96-h treatment with plant samples. Furthermore, the viability of the cells in the spheroids was examined at the end of the experiment (96 h treatment). The samples were stained with 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM) – a cell-permeable probe, which can be converted to a fluorescent form (5-carboxyfluorescein) by cellular esterases. All viable cells with intact membrane retain the formed fluorescent dye [25]. The results from these experiments showed inhibitory effects (Fig. 3) that were concordant with the data obtained with adherent adenocarcinoma cultures. Tumor spheroid size was significantly reduced by 96-h treatment with *M. friwaldskyanum* leaf

and flower extract (Fig. 3A). A tendency for decreased tumor spheroid size after treatment with stem extract was also evident. Staining with 5-CFDA, AM further strengthened these data. While control spheroids displayed intense fluorescence due to unaffected ability to convert 5-CFDA, AM and retain 5-carboxyfluorescein the cellular aggregates treated with *M. friwaldskyanum* extract demonstrated reduced fluorescence (Fig. 3B–E). Confirming previous results, strong reduction of fluorescent signal was observed for spheroids treated with flower and leaf extract (Fig. 3C and D). The extract derived from *M. friwaldskyanum* stems influenced tumor spheroid viability to a lower extent (Fig. 3E). The observed fluorescent staining was more intense than flower and leaf extract-treated spheroids (Fig. 3C and D). However, the intensity of the staining was weaker compared to the untreated control (Fig. 3B) demonstrating inhibitory effect for this sample as well. Altogether, tumor spheroid evaluations confirm the inhibitory potential of the studied samples and their ability to affect tumor cells on the multicellular level.

3.2. Influence on bacterial growth and vitality

The antibacterial properties of *M. friwaldskyanum* extracts were evaluated using one Gram-negative and one Gram-positive bacterial species – *E. coli* and *B. cereus*, respectively. After 24-h treatment with *M. friwaldskyanum* extracts the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined (Table 1). Only the leaf extract showed lower than 1000 μg/mL MIC against *B. cereus*. The other samples, as well as all samples tested against *E. coli* showed MIC and MBC of 1000 and > 1000 μg/mL, respectively. These data point to antibacterial potential of the extracts, but compared to control antibiotics, the activity of the extracts is significantly weaker.

Our experience showed that the agar disc diffusion methodology is limited and not applicable to complex samples containing substances that cannot diffuse in solid medium. Thus, to further analyze and prove the antibacterial properties of *M. friwaldskyanum* we performed longitudinal evaluation of cellular viability in liquid culture (Fig. 4). Four cultures of every bacterial species were evaluated for a total period of 36 h – untreated control grown in standard medium without addition of *M. friwaldskyanum* sample, culture maintained in medium containing flower extract, bacteria grown in medium with leaf extract, and cells cultured in medium containing *M. friwaldskyanum* stem extract. All plant samples were added to the cultures in concentration of 400 μg/mL in order to test if lower than MIC concentration could longitudinally affect bacterial survival. The viability of bacterial cultures was defined based on their ability to metabolize MTT at four time-points – 2, 12, 24 and 36 h. These experiments demonstrated potential of all *M. friwaldskyanum* samples to reduce significantly the viability of both Gram-positive and Gram-negative bacterial types for different period of time (Fig. 4). In fact, *E. coli* appeared to be more sensitive to the plant extracts because the three treated cultures showed reduced viability at 12-, 24-, 36-h' time-points (Fig. 4A). These data demonstrate sustainable effect on bacterial metabolic activity for a long period of time. *B. cereus* viability was not significantly affected at the end of the experiment and only the leaf extract induced marked reduction at the 24-h treatment (Fig. 4B). Notable reduction of *B. cereus* viability was detected 12 h after the start of the treatment, which proves the ability of *M. friwaldskyanum* extracts to suppress Gram-positive bacterial species development. However, these effects were temporary – significant reduction of bacterial viability was not detected after 36 h treatment suggesting the need for higher treatment concentrations. On the other hand, notable tendency although not significant for reduced viability was evident at 24 h and 36 h for the stem and leaf extract. Based on these experiments we were able to prove the antibacterial activity of *M. friwaldskyanum* and support future search for bactericidal compounds.

3.3. M. friwaldskyanum extracts affect the levels of important immune cell populations

To investigate the immunomodulating abilities of *M. friwaldskyanum* extracts, white blood cells derived from healthy individuals were incubated *ex vivo* with plant samples. Two extracts were chosen for these experiments – the flower sample that showed the highest antitumor potential and the stem sample that displayed the lowest level of toxicity against normal cells during the NRU assays and a different pattern of antitumor activity compared to the other two samples. Isolated leukocytes were seeded on cell culture plates and incubated for 24 h with 200 μg/mL M. *friwaldskyanum* stem or flower extracts, which was followed by immunophenotyping. The flow cytometry analyses results are present in Fig. 5 (A,B,C,D,E,F). Generally, the stem and the flower extract induced similar effects – the 24-h treatment with both samples resulted in increased levels of $CD19^+$ B-lymphocytes, CD11b-expressing cells and HLA^+ leukocytes (Fig. 5B,D,F). A tendency for increased percentages of $CD4^+$ T-lymphocytes is evident for treatment with either flower or stem extract (Fig. 5A). However, only white blood cells treated with flower extracts demonstrated significantly increased population of $CD25^+$ lymphocytes (Fig. 5E). Interestingly, the total CD25-expressing lymphocyte fraction was elevated while there wasn't a difference in $CD4^+CD25^+$ cells suggesting increase in the regulatory B-cell population. These results indicate versatile immunoregulatory properties

Table 1
Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) (μg/mL) of *M. friwaldskyanum* extracts.

Samples	*B. cereus* MIC (MBC) μg/mL	*E. coli* MIC (MBC) μg/mL
M. friwaldskyanum (flower)	1000 (>1000)	1000 (>1000)
M. friwaldskyanum (leaf)	500 (>1000)	1000 (>1000)
M. friwaldskyanum (stem)	1000 (>1000)	>1000 (>1000)

Antibiotic control (ceftrianxone): *B. cereus* – 7.5 [15] μg/mL; *E. coli* – 7.5 [26] μg/mL.

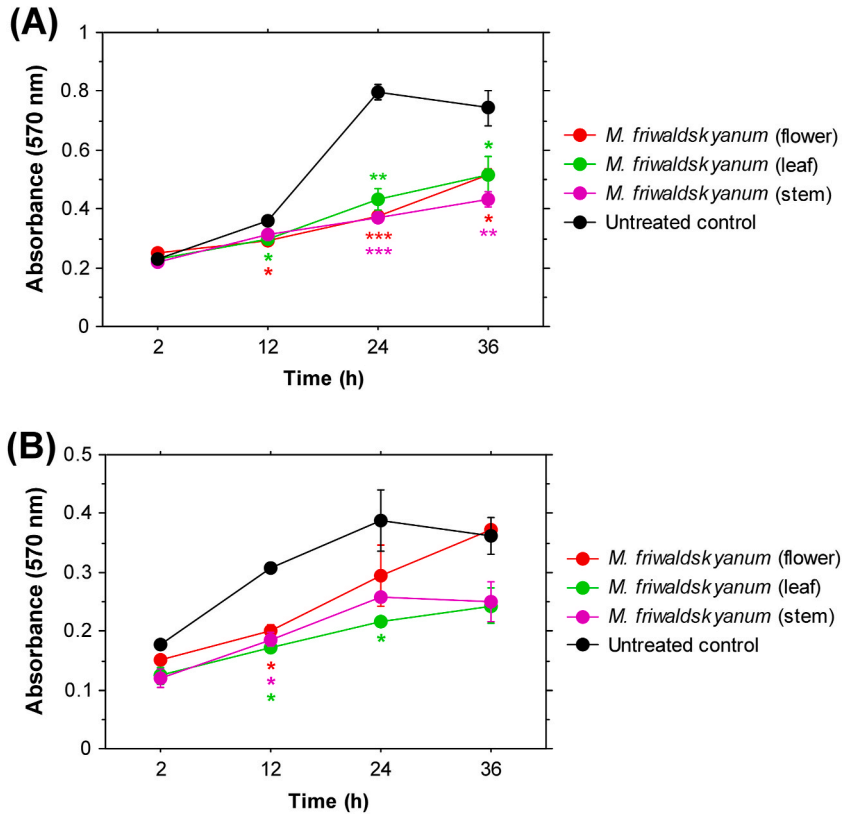

Fig. 4. Longitudinal evaluations of bacterial viability following treatment with *M. friwaldskyanum* extract. Results for *E. coli* (**A**); data for *B. cereus* cultures (**B**). The charts represent mean results (±SEM) of triplicate samples.

of *M. friwaldskyanum* and ability to modulate different lymphocyte populations and antigen-presenting cells.

4. Discussion

Plants are indispensable source of natural compounds with various pharmacological properties and important benefits for human health [13]. The use of herbs as remedies dates from antiquity and continues to be the major goal of traditional medicine [27,28]. Moreover, many contemporary drugs with critical medical importance are based on natural products with plant origin [29,30]. Analyses of plant bioactive compounds, design and synthesis of similar to herbal natural substances or modified derivative structures are fundamental for the next-generation innovative drug discovery approaches [13]. Therefore, it is not surprising that the search for new species with pharmacological significance is growing. The aim is to identify new drug candidates and/or to find a better plant source of biologically active compounds allowing cultivation and industrial production. In this regard, endemic plants have been shown to generate unique secondary metabolites and could be producers of still unidentified chemical constituents with pharmacological potential [14]. However, apart from determining specific phytochemical profile the restricted distribution limits extensive studies of endemic species. The prospect for cultivation of endemics with good reproductive potential makes them important object for future biological and pharmacological research. All these facts motivate the object of the present study – the Bulgarian endemic *Marrubium friwaldskyanum*. Phytochemical studies have indicated this species as producer of a number of biologically active natural compounds [16,19] but its biomedical potential has not been clarified. Particularly, the plant material used in the present study were previously subjected to metabolome analyses that demonstrated significant levels of diverse bioactive compounds [19]. Another reason for these studies is the reported good reproductive capacity of *M. friwaldskyanum* [16] that opens the way for introduction into cultivation and industrial use of the plant.

Extracts obtained from *M. friwaldskyanum* flowers, leaves and stems were used in the current study. These samples represent complex combination of different chemical constituents [19]. Such research material is not preferred by modern pharmacology which relies on identification of specific compounds with biomedical potential. However, a major benefit of plant extracts is the ability to induce better therapeutic effects due to unique synergistic and simultaneous activity of several compounds which individually could not exert medical properties [13]. In addition, herbal extract synergistic potential can lead to improvement of the effectiveness of standard medicines like antibiotics, chemotherapeutic agents etc., based on the combined action of the plant sample and the drug [26]. In fact, many medicinal plant extracts are currently used as prescription drugs in many developed countries [31]. Considering the complex nature of cancer diseases, infectious and degenerative disorders, it is not surprising that effective medicines cannot be

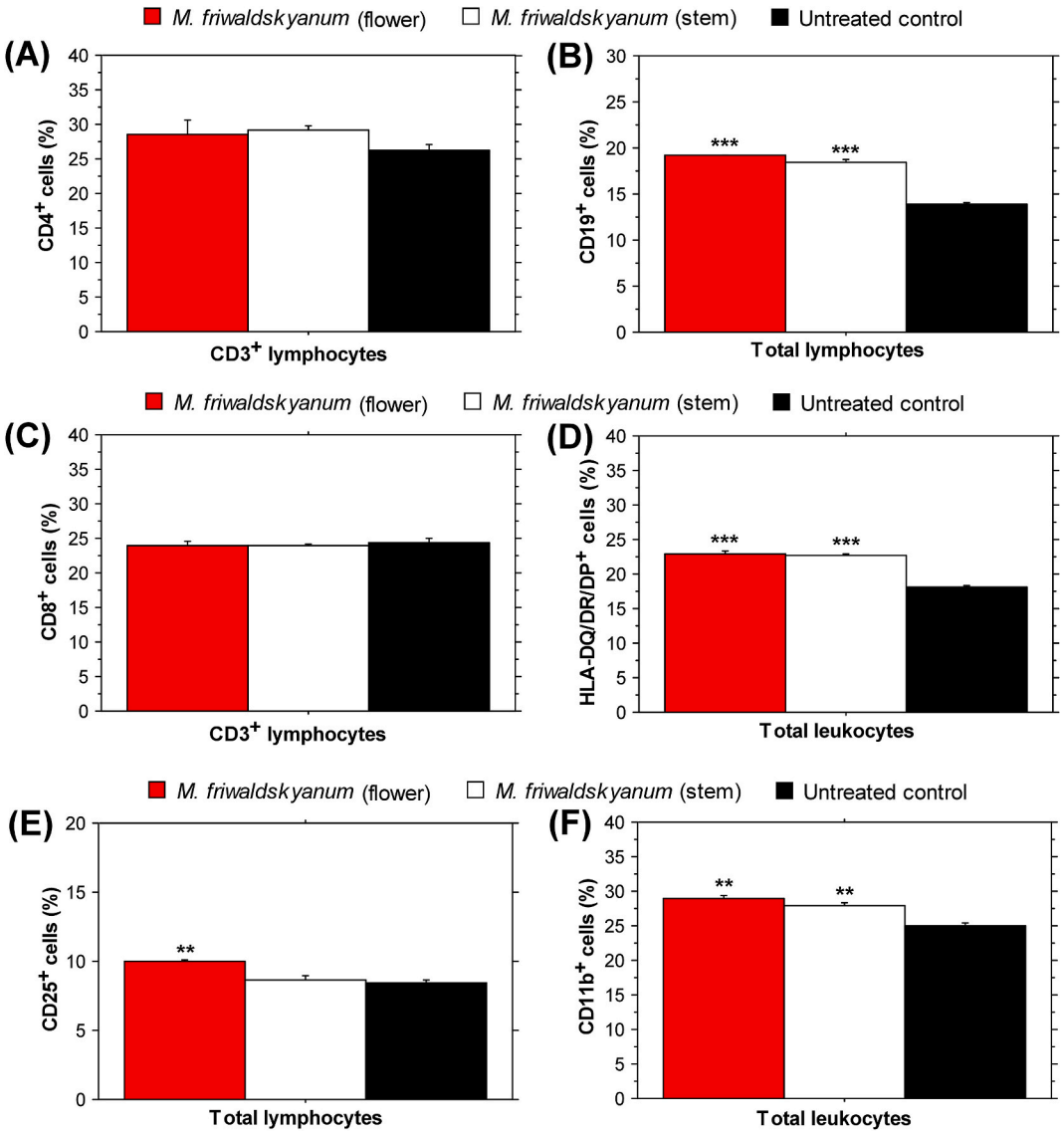

Fig. 5. Changes in main leukocyte populations following treatment with 200 μg/mL M. *friwaldskyanum* extracts. (**A**) Level of $CD4^+$ lymphocytes in the test-samples; (**B**) $CD19^+$ lymphocytes subsets; (**C**) $CD8^+$ lymphocyte levels; (**D**) Percentages of cells expressing HLA-DQ/DR/DP; (**E**) $CD25^+$ lymphocyte numbers; (**F**) Levels of CD11b-expressing leukocytes. Data are presented as the mean ± SEM of triplicates. The statistical significance of differences between control and treated leukocytes is shown as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

provided by single compound-based drug discovery. Research on plant extracts allows scientists to determine a potential therapeutic effect, which is then followed by identification of specific molecular mechanism. All these facts motivate the use of extracts as research object in this study, which demonstrates a multilateral biomedical potential of *M. friwaldskyanum* including antitumor, antibacterial and immunomodulating properties. All three analyzed extracts showed antitumor activity but the flower and the leaf sample demonstrated the highest ability to inhibit tumor cell metabolism and vitality that were confirmed on the multicellular level using HT-29 spheroids. These results are not surprising considering the metabolome profiling of *M. friwaldskyanum* [19]. Generally, the aerial parts of the plant contained significant levels of phenolic acids, flavonoids and their glucosides. Isorhamnetin, apigenin and rutin derivatives, quercetin, kaempherol, flavonoid glycosides (forsythoside, calceolarioside, leucosceptosid and their derivatives) were present in different proportions in the stems, leaves and flowers of the herb. However, unique metabolite content was demonstrated for the flowers and leaves of *M. friwaldskyanum*. Luteoloside was specifically detected in the flowers of *M. friwaldskyanum* [19]. This flavonoid possess diverse biological activities including strong antitumor potential [32].The better properties of the extract obtained from flowers could be also attributed to the reported higher levels of chlorogenic acid, apigenin, kaempherol and quercetin – compounds that are well known for their antitumor properties [33–36]. Specifically abundant in *M. friwaldskyanum* leaves were isorhamnetin–3-*O*-rutinoside and isorhamnetin–3-*O*-glucoside [19]. Based on the proven antitumor activity of isorhamnetin [37] it could

be suggested that these glucosides could contribute the more prominent effect of the leaf extract in comparison with the sample from stem. The defined antitumor effects were selective and directed against cervical and colon adenocarcinoma cells. Lung adenocarcinomic A549 cells were the least affected tumor cells but the observed inhibition was superior compared to normal fibroblasts. These data supplement previous findings regarding inhibitory potential of *M. friwaldskyanum* polyphenolic fraction against melanoma cells [20]. Furthermore, the present report shows pioneer findings with tumor spheroids that strengthen the data from adherent cultures and demonstrate ability of *M. friwaldskyanum* to inhibit the viability and growth of multicellular tumor aggregates.

M. friwaldskyanum extracts exhibited antibacterial activity against one Gram-negative and one Gram-positive species. The determined MIC and MBC values were similar to the ones measured for other *Marrubium* herbs – *M. peregrinum*, *M. incanum, M. candidissimum, M. thessalum*, *M. rotundifolium* [12,38]. Regarding the *E. coli* strain tested in the present study, *M. peregrinum* methanol extract demonstrated markedly weaker activity [39]. All three *M. friwaldskyanum* extracts demonstrated ability to significantly reduce *E. coli* viability for up to 36 h. These data support the antibacterial potential of the samples but also suggest ability to supplement drug treatment and even act synergistically to fight antibiotic resistance which is a major healthcare problem nowadays. Further investigations are needed to confirm this hypothesis particularly for *M. friwaldskyanum* samples. The potential for such synergistic action of plant extracts and antibiotics have recently been demonstrated [26].

The present study can be defined as pioneering because it reports for the first time selective antitumor activity of *M. friwaldskyanum* extracts and their antibacterial properties. Another important part of our investigations includes results for immunomodulatory potential of the studied samples. These type of studies have not been reported to date for *Marrubium* species. There are published data on the immunomodulatory potential of isolated labdane diterpenes from *M. cylleneum* and *M. velutinum* based on different methods (measurements of lymphocyte proliferation, natural killer (NK) cell or lymphokine-activated killer (LAK) cytotoxicity) [40]. Anti-inflammatory and immunomodulatory properties of *M. friwaldskyanum* can be suggested based on the presence of a number of bioactive compounds in the aerial parts of the plant [19]. Particularly, a marker substance for *M. friwaldskyanum* flowers is taurochenodeoxycholic acid [19] which possess characteristic anti-inflammatory and immunomodulatory properties [41]. Furthermore, the metabolic fingerprint of *M. friwaldskyanum* stems includes marked levels of other compounds with immunologic activity – alyssonoside [42] and salidroside [43]. In our studies, *ex vivo* treatment of white blood cells with *M. friwaldskyanum* flower and stem extracts led to notable increase in the levels of CD25, CD11b, CD19 and HLA-positive populations, which indicates modulation of both lymphoid and myeloid lineage-derived immune cells. The elevated levels of $CD19^+$ B-lymphocytes and HLA-expressing cells suggest an effect on antigen-presentation ability. Intriguingly, the total $CD25^+$-lymphocyte population was increased in the sample treated with *M. friwaldskyanum* flower extract. This result was not due to increase in the groups of activated or regulatory T-lymphocytes because we did not observe higher levels of $CD4^+CD25^+$ T cells. We speculate that this specific result is based on elevated CD25 expression by $CD19^+$ B cells and hence, an increase in the regulatory B cell population that is responsible for modulation of T-cell responses [44,45]. This would point to regulatory B cell-specific mechanism of *M. friwaldskyanum* flower extract, but additional experiments are needed to confirm this assumption.

Notably, treatment with *M. friwaldskyanum* flower and stem extracts enhanced the numbers of HLA^+ and $CD11b^+$ leucocytes. HLA molecules play a major role in antigen presentation and immune responses. Recently, it has been shown that increased HLA expression contributes to improved antitumor immunity and was associated with better outcome for patients with melanoma [46]. CD11b is an integrin and C-type lectin receptor expressed on the surface of many leucocytes (monocytes, neutrophils, macrophages, natural killer cells) and involved in regulation of cell adhesion and migration during inflammatory responses. As a C-type lectin receptor CD11b recognizes and initiate innate immune responses against pathogen-associated molecules. In addition, it was found that CD11b is responsible for macrophage polarization to proinflammatory type involved in innate antitumor immunity and suppression of tumor growth [47]. This integrin molecule is also involved in interactions with specific carbohydrates that increase the effectiveness of antitumor immunotherapy and/or induce antitumor T cell immune responses [48,49]. Thus, increased by *M. friwaldskyanum* treatment expression of CD11b and HLA molecules could contribute to improved innate and adaptive immunity status and antitumor responses.

5. Conclusions

The present report demonstrates for the first time versatile biological activities of *M. friwaldskyanum* extracts derived from flowers, leaves and stems. The antitumor, antibacterial and multilateral immunomodulatory properties could be utilized in future for generation of improved pharmacological products.

Ethical statement

The experiments were performed in accordance to the guidelines of the Declaration of Helsinki. The study gained approval by the Local Ethical Committee at "Paisii Hilendarski" University of Plovdiv, Bulgaria (protocol No. 5 from June 10, 2020). The procedure for collection of blood samples was performed in accordance with The Code of Ethics of the World Medical Association for experiments involving humans. All four volunteers were informed about the purpose of the experiments and signed a written consent before blood sampling and the start of the study.

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All authors agree to publish.

Data availability statement

Data are contained within the article or available from the corresponding author upon request.

CRediT authorship contribution statement

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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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