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Phytochemical screening, anti-herpes simplex virus, and antibacterial activities of various fractions obtained from *Graptopetalum paraguayense* E. Walter extract

Nadezhda Markova[1]*, Maya Margaritova Zaharieva[2], Petia Genova-Kalou[3], Stefka Krumova[3], Ivayla Dincheva[4], Ilian Badjakov[4*], Lyudmila Dimitrova[2], Venelin Enchev[5] and Hristo Najdenski[2]

[1] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[2] *Department of Infectious Microbiology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[3] *Department of Virology, National Centre of Infectious and Parasitic Diseases, 1233 Sofia, Bulgaria*
[4] *Department of Agrobiotechnologies, Agrobioinstitute, Agricultural Academy, 1164 Sofia, Bulgaria*
[5] *Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
**Corresponding authors:* ibadjakov@gmail.com; nadya@orgchm.bas.bg

Abstract

Markova, N., Zaharieva, M. M., Genova-Kalou, P., Krumova, S., Badjakov, I., Dimitrova, L., Enchev, V. & Najdenski, H. (2025). Phytochemical screening, anti-herpes simplex virus, and antibacterial activities of various fractions obtained from *Graptopetalum paraguayense* E. Walter extract. *Bulg. J. Agric. Sci., 31*(5), 997–1011

Our previous studies have demonstrated that the methanol/water extract of *Graptopetalum paraguayense* E. Walter (GP) significantly inhibits herpes simplex virus type 1 (HSV-1) and exhibits moderate activity against Gram-positive bacteria. We conducted a metabolic profile of the plant, yielding three fractions (A-polar metabolites, B-fatty acids, sterols, tocopherols, and C-phenolic components), which were analyzed using GC-MS. This study tested these fractions for cytotoxic, antibacterial, and anti-HSV activities *in vitro*. Cytotoxicity was tested on the Vero cell line. The HSV strains tested included two wild-type (*wt*) strains sensitive to acyclovir (ACV), Victoria (HSV-1) and Bja (HSV-2), and two clinical isolates resistant to ACV (ACV^R), DD (HSV-1) and PU (HSV-2). Antibacterial activity was tested against the following bacterial strains, as per ISO Standard 20776-1: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* 25922, and *Pseudomonas aeruginosa* ATCC 27853. Fraction C from GP selectively inhibited HSV-1 Victoria replication at 0.01 mg/mL, offering 94.5% cell protection. Its antiviral effect against acyclovir-resistant HSV strains was low (25.5% cell protection), with IC_{50} values ranging from 0.01 to 0.1 mg/mL, but superior to that of ACV (10.8% cell protection). This fraction demonstrated activity against *Staphylococcus aureus* ($MIC_{\text{Fraction C}}$ = 0.625 mg/mL) and inhibited methicillin-resistant *S. aureus* biofilm formation by 50%-90%. Phenolic fraction was non-cytotoxic and caused no skin irritation in rabbits, making it a promising candidate for antiviral drug development against HSV-1 and topical treatment for Gram-positive bacterial infections.

Keywords: Graptopetalum paraguayense E. Walter; anti-HSV activity; antibacterial effect; minimal inhibitory concentration (MIC); effective concentration 50% (EC_{50}); cytotoxicity

1. Introduction

The rising risk of emerging and re-emerging infections in the human population has become a significant global concern of the twenty-first century, due to their substantial health and economic impacts (Woolhouse et al., 2001). The use of antiviral and antibacterial drugs increases alongside the development of vaccines, facilitating the effective control of infectious diseases (De Clercq and Li, 2016; Lewis, 2017). However, systemic application of these agents is often limited by the development of drug resistance (Frobert et al., 2008; Van Boeckel et al., 2014) or toxicity (Monto, 2006), especially in immunosuppressed HIV-infected and post-transplant patients (Dumford and Skalweit, 2016; Limaye, 2002; Singh et al., 2020), which highlights the need for new therapeutic options. In this context, the development of alternative antibacterial and antiviral agents is urgently required for the management and control of viral and bacterial infections when vaccines and standard therapies are lacking. Herbal medicines and purified natural products represent a rich resource for the development of novel antimicrobial drugs (Lin et al., 2014; Raskin et al., 2002; Wright, 2014). Many articles describe the screening and testing of major groups of bioactive plant compounds, such as alkaloids, flavonoids, phenolic acids, polyphenols, tannins, terpenoids, saponins, many of which exhibit radical scavenging, antiviral, and antibacterial properties (Chassagne et al, 2021; Parham et al., 2020). The great diversity of plant extracts as a potential source for new drugs provides numerous advantages, such as reduced side effects, less resistance, low toxicity to cells, and various mechanisms of action compared with standard chemotherapy(Raskin, et al., 2002; Vaou et al., 2021).

Most of the active plant components in members of the *Crassulaceae* family have antioxidant, anti-inflammatory, nd a broad spectrum of antimicrobial activities against food-borne pathogens and spoilage bacteria (Hassan et al., 2021; Xu et al., 2018). In particular, the presence of hydrophilic functional groups, such as the hydroxyl groups of phenolic components and/or the lipophilicity of some essential oil components, has a greater inhibitory effect against Gram-positive bacteria than against Gram-negative bacteria (Lucera et al., 2012; Nwadinigwe, 2011). Selected succulents, including *Graptopetalum mendozae* (*Crassulaceae*), possess antibacterial properties against Gram-negative bacteria such as *A. hydrophila* and *V. harveyi*. The MIC and MBC results for *V. harveyi* showed that all extracts were effective in inhibiting the bacterium at 0.25 g/mL, except for the ethanol extract of *G. mendozae* at 0.125 g/mL (Ordanel et al., 2023).

Many members of the *Crassulaceae* family exhibit antiviral effects, including anti-HSV (Cryer et al., 2017; Kandar, 2022; Ürményi et al., 2016). The selection of the herpes virus type 1 (HSV-1) and type 2 (HSV-2) were based on the clinical importance of these infections within the human population (Fatahzadeh and Schwartz, 2007), the appearance of drug-resistance strains or toxicity (Bacon et al., 2002), especially among immunocompromised individuals (Celum, 2004). The development of therapeutic agents has become necessary due to the increasing incidence of diseases. Many phytochemicals possess antiviral activity against HSV types such as flavonoids (Lin et al., 1999), alkaloids (Martin, 1987), saponins, terpens, tannins, polysaccharides (Bourne et al., 1999), thiosulfinates (Tsai et al., 1985), steroidal glycoside (Ikeda et al., 2000), lignans (Charlton, 1998), quinones (Andersen et al., 1991) and proanthocyanidins (Erdelmeier et al., 1996). It was revealed that their important functions are inhibiting different stages of viral replication (El-Toumy et al., 2018), viral genome synthesis (Lin et al., 2011), host-cell receptor attachment (Rajbhandari et al., 2001), and intercellular junction spread of HSV-1 (Abad et al., 2000).

Graptopetalum paraguayense E. Walther (GP) is a traditional Chinese herbal medicine belonging to the *Crassulaceae* family. The plant is succulent, mainly cultivated as an ornamental houseplant /worldwide and used in Taiwanese folk medicine. However, the GP is highly promising from a pharmacological perspective (Eid et al., 2018). It was found that GP aqueous extracts exhibit anti-inflammatory and hepatoprotective (Chen et al., 2016), antioxidant (Chung et al., 2005) properties, potential therapeutic agent for Alzheimer's disease (Wu et al., 2019), anti-hypertensive and antihyperglycemic (Yen et al., 2013), immunomodulatory (Kim et al., 2006), antineoplastic (Hsu et al., 2015), antiviral (Jhuang et al., 2015; Todorova et al., 2022; Zaharieva et al., 2019) and antibacterial (Zaharieva et al., 2019) activities. Recently, it was discovered that the 50% ethanol extract of GP may have a potential cytotoxic effect on human melanoma, leading to G2/M phase arrest, ER stress, and intrinsic apoptotic pathways (Peng et al., 2023). Another recent study demonstrated that the aqueous extract of GP enhanced antioxidant activity and protected against methylglyoxal-induced inflammatory liver injury in rats, as well as against high-fructose diet-induced intestinal dysbiosis in mice (Lee et al., 2023). In our preliminary study (Zaharieva et al., 2019), we demonstrate for the first time the cytotoxic, anti-herpes simplex virus (anti-HSV), and antibacterial activities of a total methanol/water extract of GP. We found that a total GP extract showed a significant inhibitory effect on the wild-type herpes simplex virus type 1 (HSV-1) strain Victoria in a concentration of 0.0001 mg/mL, equal to the maximal nontoxic concentration (with 97.5% protection of the cells). To explain this vigorous antiviral activity, we conducted a metabolic analysis of the plant (Todorova et al., 2022). Three main fractions – non-polar substances, polar metabolites, and phenolic compounds – were obtained, and GC-MS analysis was carried out. These substances, identified by GC-MS analyses, were utilized in molecular docking simulations and quantum-chemical calculations to determine the binding affinity of the plant compounds to the HSV DNA polymerase active site. According to the interaction energies of all five ligand-amino acid complexes, the hydrogen bonding in all of them is strong.

In our previous study (Zaharieva et al., 2019), we demonstrated that a total extract of *G. paraguayense* exhibits moderate antibacterial activity including inhibition of the bacterial growth and the biofilm formation in methicillin resistant *Staphylococcus aureus*. The antibacterial spectrum of the total extract covered Gram-positive bacteria. However, it remained unclear which fractions or phytochemical groups of compounds contribute to the extract's antimicrobial potential. Chao et al. (2019) found that a 50% ethanolic extract from *G. paraguayense* exerted a vigorous antioxidant activity. Determination of its chemical composition by HPLC revealed high amounts of isoflavones and quercetin, which explains the high radical scavenging potential found. Antibacterial properties often accompany vigorous antioxidant activity; therefore, it is worthwhile to search for fractions isolated from *G. paraguayense* extracts with such potential.

To our knowledge, data concerning the antiviral and antimicrobial potential of GP, including its chemical composition, have not been reported. Thus, in the present study, we aim to investigate the antibacterial and anti-herpes activities of three main fractions obtained from metabolome analysis. Polar metabolites, fatty acids, sterols, tocopherols, and phenolic components, isolated from aerial parts of *G. paraguayense* E. Walther, will be tested on cell cultures.

2. Materials and Methods

2.1. Plant material and GP extracts preparation

2.1.1. Plant material

G. paraguayense E. Walter was grown as an ornamental plant at the Institute of Organic Chemistry with Centre of Phytochemistry, Sofia, Bulgaria. Botanical identification and authentication were performed by Asen Asenov, PhD (Sofia University 'St Kliment Ohridski') and Antoaneta Petrova, PhD (Botanical Garden, Bulgarian Academy of Sciences), and a voucher specimen number SO 107 621 was deposited in the herbarium of Sofia University, Bulgaria.

2.1.2. Chemicals

All chemicals (MeOH, $CHCl_3$, NaOH, H_2SO_4, Methoxyamine hydrochloride, HCl, EtOAc, *n*-Hexane, BSTFA, Pyridine) were of HPLC grade (Sigma-Aldrich, St. Louis, MO), and water was of Milli-Q (18 MΩ/cm) (Millipore Corp., Bedford, MA).

2.1.3. Extract preparation

Leaf extract was prepared as described in our previous study (Zaharieva et al., 2019) and (Todorova et al., 2022). According to the described procedure, three main fractions were isolated: fraction A includes non-polar metabolites, such as fatty acids, sterols, and terpenoids; fraction B consists of polar metabolites, including amino acids, sugars, and other organic acids; and fraction C includes hydroxybenzoic acids.

Fractions A, B, and C were subjected to further analyses.

GC-MS sample preparation and GC-MS analyses

GC-MS analysis was carried out as described in our previous studie(Zaharieva et al., 2019) and (Todorova et al., 2022).

2.2. Evaluation of anti-HSV activity of leaf extract of G. paraguayense E. Walther in vitro

2.2.1. Cells and virus strains

Vero (African green monkey kidney cell line) were obtained from National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria and cultured in Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 µg/mL streptomycin, 100 IU/mL penicillin, two mM L-glutamine, 4.5 g/L glucose (Sigma-Aldrich, Germany) (growth medium) (GM) at 37°C in a 5% CO_2 incubator for 72 h. The infection medium (IM), used for the antiviral assays, was the same as the GM, but 2% FBS was added.

The following HSV strains (NCIPD collection) were used: two wild-type (*wt*) strains sensitive to ACV, Victoria (HSV-1) and Bja (HSV-2), and two clinical isolates resistant to ACV (ACV^R), DD (HSV-1) and PU (HSV-2). Virus titers were determined by the virus-induced cytopathic effect (CPE) in Vero cell culture and expressed as 50% tissue culture infective dose per mL ($TCID_{50}$/mL) using the Reed and Muench method. A 100 $TCID_{50}$ was used for the assay (Reed & Muench, 1938). All virus stocks were stored at −70°C until used. The anti-herpes drug ACV (Sigma-Aldrich, USA) (1 mg/mL) was used as the standard.

Preparation of stock solution of GP fractions

The obtained GP fractions were first dissolved in 1 mL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) and filtered through a sterile syringe filter (0.2 **µm pore diameter) to obtain stock solutions (SS). The SS was diluted with the maintenance medium (culture medium** containing only 2% FBS) to obtain a final concentration of 2 mg/mL (0.1% DMSO).

2.2.2. Cytotoxicity assay

The cytotoxicity of the GP fractions A, B, and C was tested on Vero cells and was determined by microscopic observation of monolayers (Serkedjieva and Hay, 1998) and by a colorimetric MTT assay (Mosmann, 1983) with minor modifications (van Meerloo et al., 2011).

2.2.3. MTT assay

The MTT assay was based on the metabolic reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, Germany) by the mitochondrial enzyme activity of viable cells into an insoluble, colored formazan product (dark purple), which can be measured optically. Cells were seeded (0.5 x 10^5 cells/100 µL/well) in flat-bottom 96-well tissue culture plates (Corning Inc., USA). Following 24 hours of incubation, the cells were treated (in triplicate) with various concentrations of test fractions (0.1–1.5 mg/mL) and the drug ACV (0.0005–0.1 mg/mL), both prepared in culture media, and incubated at 37˚C for 72 hours. The final concentration of DMSO never exceeded 0.1% in any of the assays and therefore, had no signs of toxicity. Blank (only media) and untreated/negative (0.1% DMSO in media) controls were also included. Cells were treated with MTT reagent (10 µL/well) and further incubated at 37˚C for 3 4 h. Upon appearance of a purple color, the detergent solution (DMSO & EtOH) (100µL) was added to each well and further incubated at 37˚C for one h. The optical density (OD) was recorded at $\lambda = 540$ nm by an ELISA reader (ELx800; BioTek Instruments Inc., USA). The percentage (%) of viable treated cells was calculated using the formula:

$$\% \text{ cell viability} = (OD_{\text{treated cells}} - OD_{\text{blank/negative control}} / OD_{\text{cell control}} - OD_{\text{blank/negative control}}) \times 100$$

The results reported are the mean values of two separate experiments, each performed in triplicate.

CC_{50} was the concentration required to reduce the OD or to induce visible morphological changes in 50% of cells. The maximum nontoxic concentration (MNC) was defined as the minimum dilution of the fraction, which did not cause a toxic effect or death of the treated cells.

2.2.5. Microscopy (Simões, Amoros, & Girre, 1999)

Vero cell cultures (2 × 10^^{5} cells/ml) were prepared in 96-well tissue culture plates (Corning, US). After a 24 h period of incubation at 37°C in a humidified 5% CO_2 atmosphere, cell monolayers were confluent. The medium was then removed from each well and replaced with different concentrations of the tested GP fractions. For cell control, 200 µl of cell culture medium without substances was added and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cell morphology was observed daily (24h, 48h, and 72h) for microscopically detectable morphological alterations, such as loss of confluency, cell rounding and shrinking, and cytoplasmic granulation and vacuolization, under an inverted microscope (Bio Optical, Italy) at a magnification of x200. Morphological changes were scored, and MNC and CC_{50} values were estimated.

2.2.6. Anti-HSV activity assay

The virus-inhibitory effect of the GP fractions A, B, and C was determined by two assays: the cytopathic reduction assay (CPE-reduction assay) and the dose-dependent analysis of HSV replication by MTT assay.

2.2.6.1. Cytopathic effect reduction assay (CPE-reduction assay) (El-Toumy, Salib, El-Kashak, Marty, Bedoux, & Bourgougnon, 2018)

Vero cells (0.5 × 10^^{5} cells/well) were seeded and incubated at 37°C in a 5% CO_2 atmosphere until the cells became confluent. Thereafter, the culture medium was removed from each well, and 0.1 mL of virus suspension containing 100 TCID50/well and 0.1 mL of DMEM containing 2% FBS were mixed in each well of 96-well plates, and appropriate concentrations of the extract from minimal to maximal non-cytotoxic concentration were added to each well based on serial dilution preparation. Plates were examined at 72 h by inverted light microscopy for cellular changes/CPE on Vero cells. The reduction of virus multiplication was calculated as a percentage of virus control ($CPE_{exp}/CPE_{\text{virus control} \times}$ 100). The concentration of the tested fraction, which reduced CPE by 50% compared to the virus control, was estimated from graphical plots defined as the 50% inhibitory concentration (IC_{50}), expressed in mg/mL, using modeling software. The maximal effective concentration 50% (EC_{50}) was expressed as the concentration that achieved 50% protection of cells from the HSV-induced destruction. The selectivity index (SI) was calculated as the $CC_{50}/EC_{50\ \text{ratio}}$. The selectivity index (SI) was measured from the ratio of CC_{50}/EC_{50} (Kudi and Myint, 1999).

2.2.6.1. Dose-dependent analysis of HSV replication in treated cells by MTT assay

We used a modification of an MTT assay developed for screening anti-HSV compounds by Takeuchi et al. (1991). In short, confluent Vero monolayers were overlaid with virus suspensions at a low MOI 100 $TCID_{50}$/well and non-cytotoxic (MNC and low) concentrations of GP extract (0.1 mL/well). The effect was measured at 72 hours post-infection (p.i.) as described in the cytotoxicity assay. The percentage of protection was calculated as follows: $[(OD_{exp}) - (OD_{virus\ control})]/[(OD_{cell\ control}) - (OD_{virus\ control})] \times 100$ (%), where OD_{exp}, $OD_{virus\ control,}$ and $OD_{cell\ control}$ indicate the absorbance of the test sample, the virus, and the cell controls, respectively. The EC_{50} and SI were determined as described above.

2.2.7. Statistical analysis

The GraphPad Prism software package, version 8.00 for Windows (La Jolla, California, USA; www.graphpad.com), was used to draw the dose-response curve and calculate the CC_{50} and IC_{50} of the separated GP fractions. The percentages of viral inhibition by GP fractions about each tested virus represent the mean ± standard error of the mean values of three different experiments. Experiments were performed in triplicate. Student's *t*-test was used, and $p<0.05$ was considered significant. The correlation coefficient values (R^2) were calculated.

2.3. Evaluation of antibacterial and anti-biofilm activity

2.3.1. Bacterial strains and growth conditions

The antibacterial activity of the fractions A, B, and C was tested on the following bacterial strains according to ISO Standard 20776-1: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* 25922, and *Pseudomonas aeruginosa* ATCC 27853, all purchased from the American Type Culture Collection, USA. The following bacterial strains were used not only in the broth microdilution (BMD) test but also for evaluation of biofilm and redox inhibitory activities: methicillin-resistant *S. aureus* (MRSA) NBIMCC 8327 (National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria). The strains were maintained in Trypticase Soy Agar/Broth (TSA) (Himedia, India) and TSB. The BMD assay was performed in Mueller Hinton broth (MHB, Thermo Scientific-Oxoid, UK). The biofilm experiment was conducted in BHI, supplemented with 2% D-glucose (Sigma Life Science, Germany). The bacteria were cultured in an incubator at 37°C.

2.3.2. Broth microdilution test

The antibacterial activity of the tested fractions was evaluated according to ISO 20776/1-2006 ("ISO20776/1-2006, Clinical laboratory testing and in vitro diagnostic test systems – Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices – Part 1: Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases, 2006, p.19.," 2006) based on the BMD test. Twofold serial dilutions of the extracts, ranging from 5 to 0.3125 mg/ml, were prepared in triplicate in 96-well plates (final volume of 50 µl) for all bacterial test strains. An equivalent volume of bacterial suspension ($5 \times 10^{\wedge 5}$ CFU/mL) was added to each well. The plates were incubated at 37°C for 24 h. The lowest drug concentrations, which inhibited visible bacterial growth, were considered the minimal inhibitory concentration (MIC). Gentamycin (0.008-4 mg/L) and penicillin (0.008-4 mg/L) (Gibco, Life Technologies Ltd., Paisley, UK) were used as reference antibiotics (positive control). The recommendations of EUCAST (European Committee on Antimicrobial Susceptibility Testing) were followed for the analysis of the results (EUCAST, 2019). PBS served as the negative control, and MHB as the blank solution.

2.3.3. Biofilm formation assay

The biofilm formation assay was performed according to the protocol described by Stepanovic et al. (Stepanović et al., 2000). Twofold serial dilutions of the extracts, ranging from 5 to 0.3125 mg/ml, were prepared in triplicate in flat-bottom 96-well polystyrene tissue culture plates in BHI broth (Himedia, India) supplemented with 2% D-glucose (w/v) to a final volume of 100 µl/well. An equivalent volume of MRSA bacterial inoculum ($5 \times 10^{\wedge 5}$ CFU/mL) was added to each well. The culture was incubated for 24 h at 37 °C under aerobic static conditions. After discarding the supernatant, the attached bacterial cells were washed three times with PBS (200 µl/well), fixed in methanol (200 µl/well for 15 min), air-dried, and stained with 0.1% crystal violet (200 µl/well for 5 min). The stain was rinsed off with tap water, and the samples were air-dried. The biofilm formation was documented under an inverted light microscope. Thereafter, the attached stained bacteria were dissolved in 160 µL of 33% acetic acid, and the OD was measured at λ=550 nm. The minimum biofilm inhibition concentration ($MBIC_{50}$) was defined as the concentration of the tested drug that resulted in

50% inhibition of biofilm formation. The values were calculated based on the absorbance of crystal violet.

2.4. Skin irritation test

The skin irritation test was carried out in rabbits according to ISO 10993 10 (ISO 10993-10:2010, 2016). Two concentrations were used for the test – the MIC was determined for the most sensitive bacterial strain (0.625 mg/mL) and 10 times the MIC. For the test, healthy young female albino rabbits from a single strain, weighing 2.5 kg and with intact, healthy skin, were used (Permit to perform the irritation skin test on rabbits Nr.). 232 for the Animal facility with No. 1113-0005, valid until 11.04.2024, and issued by the Ministry of Agriculture, Food and Forestry, Bulgarian Food Safety Agency. The animals were acclimatized and cared for as specified in ISO 10993-2 and Ordinance No. 20, 01.11.2012 (State Gazette 87/9.11.2012). The fur on the back of the rabbit was clipped (10 × 15 cm) within 24 h before treating. Each extract dose was dissolved in 0.5 mL of distilled water (dH_2O) using sonication. The samples were applied directly to each test skin site, covered with a non-occlusive absorbent gauze patch (2.5 × 2.5 cm), and wrapped with a semi-occlusive bandage. Sodium dodecyl sulfate (SDS, 10% in dH_2O) was used as a positive control for skin irritation, and dH_2O as a negative control. After a 24 h exposure time, the patches were removed, and the rest of the test samples were washed away with dH_2O. The Primary Irritation Index (PII) was calculated based on the Primary Irritation Score (PIS) for each sample, and the results were interpreted according to the Scoring system for skin reactions.

2.5. Calculation of median inhibitory concentrations and statistical analysis

The median inhibitory concentration of the tested extract for the biofilm formation was calculated using the GraphPad Prism software (Version 5.00, for Windows, GraphPad Software, La Jolla California, USA, www.graphpad.com), using the nonlinear regression model *log(inhibitor) vs. normalized response, variable slope: Y=100/(1+10^((LogIC50-X)*HillSlope))*. All experiments were performed in triplicate. The experimental data were analyzed statistically using the two-sample independent Student’s *t*-test. Data were presented as the mean ± SD (standard deviation). A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

Common drug resistance (to acyclovir and related nucleoside analogues) in HSV strains develops after treatment (Whitley et al., 1998), and is most commonly due to mutations in HSV thymidine kinase (TK) or DNA polymerase. Therefore, new antiviral agents exhibiting different mechanisms of action are urgently needed. Plant extracts are promising and reliable resources with significant potential for anti-herpes activity. Recently, we found that the total methanol extract from *Graptopetalum paraguayense* E. Walther exhibits a significant inhibitory effect on (Zaharieva et al., 2019). To explain this strong antiviral activity, a metabolic analysis of the plant was performed. Three main fractions – non-polar substances, polar metabolites, and phenolic compounds were obtained, and GC-MS analysis was carried out (Todorova et al., 2022). According to molecular docking analyses and quantum-chemical calculations on the primary metabolites, the hydroxybenzoic acids from GP exhibit binding affinity to the HSV DNApol active site, albeit significantly weaker than that of acyclovir. In the present work, we expand the research by presenting tests for anti-herpes and antibacterial activity of the three main fractions of GP, obtained and analysed by metabolic and GS-MS analyses (Todorova et al., 2022).

3.1. Design of the study

For the metabolic analysis and investigation of the *in vitro* cytotoxicity, antiviral, and antibacterial effects of the GP fractions A, B, and C, we followed the procedure presented in Scheme 1. We selected for our study a panel of HSV-1/HSV-2 strains sensitive to ACV and resistant to ACV^R mutants, as well as a panel of pathogenic bacterial strains.

3.2. Metabolic analysis

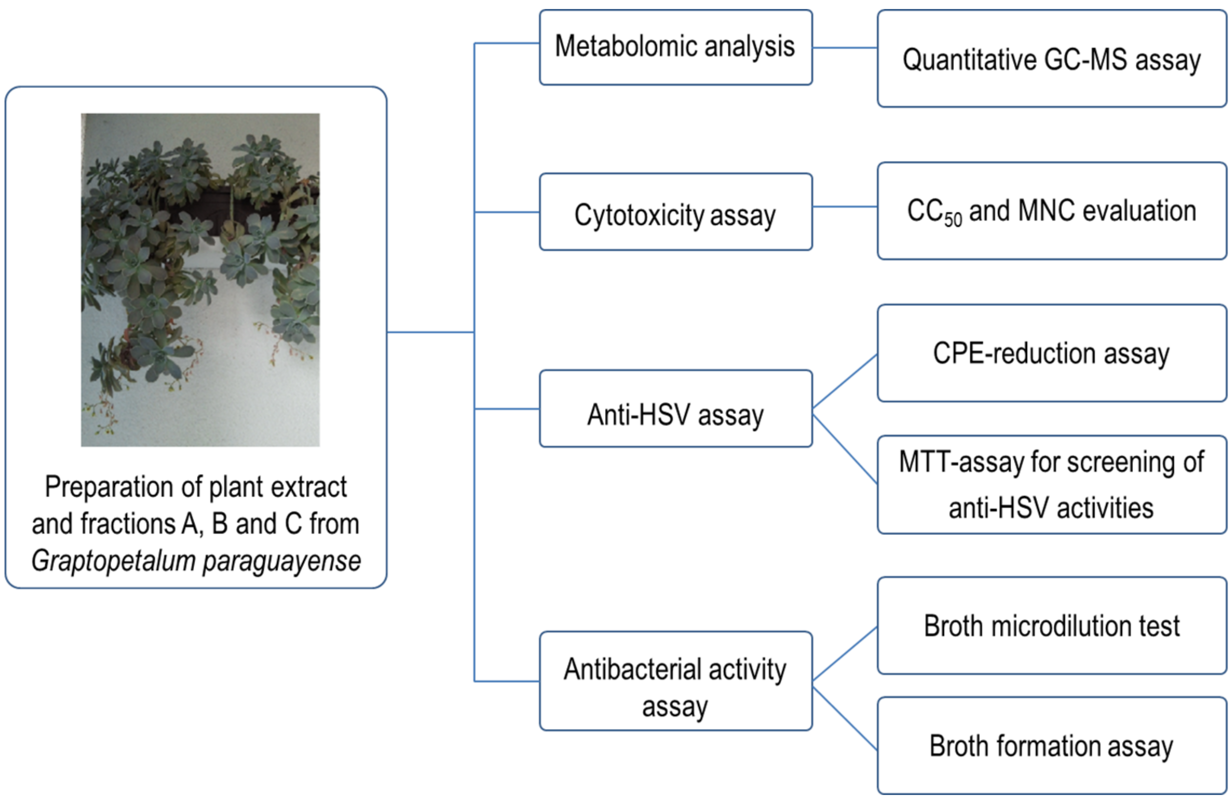
The GC-MS was used to investigate the chemical composition of the three fractions derived from GP leaves (Todorova et al, 2022). Among all the investigated fractions, it was demonstrated that fraction C is rich in various molecules of great interest. It was distinguished by the presence of 1,8-dihydroxyanthraquinone (426.83 µg/g DW), followed by syringic acid (291.36 µg/g DW), *trans*-ferulic acid (218.97 µg/g DW), gallic acid (183.27 µg/g DW), and *p*-coumaric acid (147.80 µg/g DW). Fraction B was characterized by the presence of linolenic (2842.50 µg/g DW), linoleic (2522.25 µg/g DW), and oleic (459.56 µg/g DW) acids as well as β-amyrin (2080.86 µg/g DW), β-sitosterol (2010.41 µg/g DW), and α-tocopherol (1447.18 µg/g DW). Finally, fraction A was characterized by the presence of biomolecules belonging to different chemical classes, primarily amino and organic acids, as well as mono- and dicarbohydrates. The predominant components in the essential amino acid class were leucine (92.04 µg/g DW), phenylalanine (82.72

μg/g DW), and isoleucine (80.74 μg/g DW). In contrast, the major constituents in the class of organic acids were malic (1830.54 μg/g DW) and citric (110.94 μg/g DW) acids. The classes of monocarbohydrates were characterized by mannose (1335.38 μg/g DW) and sedoheptulose (803.77 μg/g DW), while in the class of dicarbohydrates, sucrose (473.52 μg/g DW) was identified, respectively. The GC-MS chromatograms of the identified components from GP fractions are presented in Figure S1 and Tables S1-3 in the *Supplementary material*.

3.3. Cytotoxic effect of GP fractions

Cytotoxicity evaluation in cell cultures is a crucial aspect of screening potential antiviral agents (Andrighetti-Fröhner, Antonio, Creczynski-Pasa, Barardi, & Simões, 2003). Tested compounds should exhibit low toxicity against host cells, with minimal or no effects on cellular metabolism (Simões, Amoros, & Girre, 1999). The toxicity of GP fractions to Vero cells was investigated by two different methods: (i) determination of effects on cell morphology evaluation by inverted light microscopy, which is qualitative and more subjective; and (ii) the cell viability test by using the colorimetric MTT assay - the most commonly used colorimetric indicators of cell viability and it has been used to evaluate cytotoxicity in a quantitative way (Smee et al., 2002). The first step was to determine the concentration range of the tested GP fractions, starting with non-toxic concentrations (cell viability ≥ 80-90% compared to the control) and progressing to concentrations where cell viability was ≤ 40% compared to the control. Preliminary studies had already demonstrated that a total methanol/water GP extract presents a high cell-tolerable concentration rane (Zaharieva et al., 2019).

Cells were incubated with different concentrations of the plant fractions A, B, and C (ranging from 0.1 to 1.5 mg/mL) for 72 hours. Microscopic observation of the Vero cell monolayer morphology after treatment with a concentration range of 0.1–0.8 mg/mL did not reveal cytopathology typical of any toxic effect of the extract. In contrast, the viability of the Vero cells was significantly inhibited after the application of higher concentrations of GP fractions, exceeding 1 mg/mL. Similar morphological characteristics have been observed in cells exposed to ACV at concentrations ranging from 0.1



Scheme 1. Procedure for evaluation of metabolomic analysis, anti-HSV and antibacterial activities of GP fractions *in vitro*

to 1 mg/mL, where apoptosis has been previously reported (Varadinova et al., 2005). In the control group, which was treated only with DMSO, the cellular survival was calculated to be 100%. Additionally, cell survival analyses by the MTT assay indicated that the GP fractions caused growth inhibition and decreased cell viability in Vero cell lines in a dose-dependent manner (Figure 1). Dynamics of viability of the cells treated with different concentrations of tested GP fractions at 24 h, 48 h (data not shown), and 72 h were determined from the dose-response curves.

The GP fractions A, B, and C investigated decreased viability and/or proliferation of the treated cells in a time- and concentration-dependent manner. Based on the MNC data, the three GP fractions, A, B, and C, expressed the same cytotoxic potential in Vero cells. The CC_{50} values obtained by the MTT assay correlated well with the observed morphological changes in the cells. They were calculated by regression analysis of the dose-response curves generated from the data. The cytotoxic potential of the three fractions was well demonstrated by CC_{50} values, according to which they were three times less cytotoxic compared to the anti-herpetic drug ACV. The data from our experiments showed that the GP fractions could be administered in a concentration range (MNC and lower) that avoids significant cell damage.

3.4. Assessment of anti-HSV-1/HSV-2 activities of GP fractions

3.4.1. Cytopathogenic effect (CPE) reduction assay

Fractions A, B, and C were studied for the cytopathic effect against two wild types (*wt*), sensitive to Acyclovir (ACV) strains (Victoria, HSV-1 and Bja, HSV-2), and two ACV-resistant mutants (DD, HSV-1 and PU, HSV-2). After titration of the HSV-1 strain Victoria and the HSV-2 strain Bja on the Vero cell line, we determined a final cell culture titer of 10-6.5 $TCID_{50}$/mL and 10-4.5 $TCID_{50}$/mL, respectively. The titer was 10-4.0 $TCID_{50}$/mL and 10-4.83 $TCID_{50}$/mL for the two mutants HSV-1, strain DD, and HSV-2, strain PU, respectively. Subsequently, we used HSV-1 and HSV-2 strains at a concentration of 100 $TCID_{50}$/mL.

The research design refers to the overall strategy of looking for new substances capable of overcoming existing resistance to ACV. Therefore, all data obtained were compared to ACV used as a positive control in the concentration range 0.0005-0.5 mg/mL. To evaluate the effect of three GP fractions on the replication of HSV-1 and HSV-2 strains, the values of maximum nontoxic concentration (MNC) were in a concentration range of 0.00001-0.1 mg/mL. Dose-dependent curves were constructed to determine the inhibitory concentration leading to 50% inhibition of the viral cytopathic effect (IC_{50}). A lower IC_{50} value indicates more potent antiviral properties of the studied substances. Uninfected cells in the absence of plant material were used as cell control, while HBV-1 and HBV-2 infected cells in the absence of substance were used as virus controls.

Visualisation of the variations in cell monolayer morphology after treatment with plant fractions is an accessible and reliable approach in the initial study of the antiviral properties of various substances. In this regard, in parallel with the MTT analysis, changes in the morphology of infected and treated cells subjected to the effect of the fractions were observed under an inverted light microscope. Cell cultures were observed daily for the development of HSV-1 and HSV-2. CPE was expressed in the formation of giant multinucleated cells (syncytia), which were disseminated unevenly throughout the cell monolayer, with a tendency to form foci. The effects of fractions A, B, and C at 48 h were 1 to 2+ (partial depletion of the cell monolayer); therefore, CPE was reported at 72 h (3-4+ degeneration) after infection of Vero cells. In the presence of inhibition of CPE, it is concluded that not only the replication of the virus is inhibited, but also most likely the target of the inhibitory effect in different strains of HSV-1 and HSV-2 could be due to differences in the replication cycle, mutations in virus-specific enzymes such as thymidine kinase (TK), regulatory proteins, etc. In the control, uninfected with HSV-1 and HSV-2 cell cultures, no CPE effect was found. Controls infected with the HSV-1 strain Victoria at a concentration of 100 $TCID_{50}$/mL showed a rapid and pronounced CPE, noticeable even 24 h after viral adsorption, expressed in round multinucleated cells clustered as "islands", isolated from each other and separated from the substrate on which they are cultivated. On microscopic monitoring studies of the monolayer's morphology, it can be concluded that GP extract and active fraction C applied in MNC showed different degrees of CPE inhibition of HSV-1 and HSV-2-infected cell strains after 72 h of treatment. The best suppression of CPE was observed after treatment with fraction C of infected MDCK cells administered in MNC (0.01 mg/mL). The total alcoholic extract of GP observed a less pronounced CPE inhibitory effect at a concentration of 0.0001 mg/mL. When applying fraction B to Vero cell culture, we found that a noticeable destructive effect on the cell monolayer was observed with MNC (0.01 mg/mL).

On the other hand, it should be noted that all tested active fractions and the total extract of *G. paraguayense* E. Walther were administered p.i. Inhibit to a significantly lower extent the yield of infectious viral progeny of HSV-2 in cell cultures. It should be noted that the answer is strain-specific.

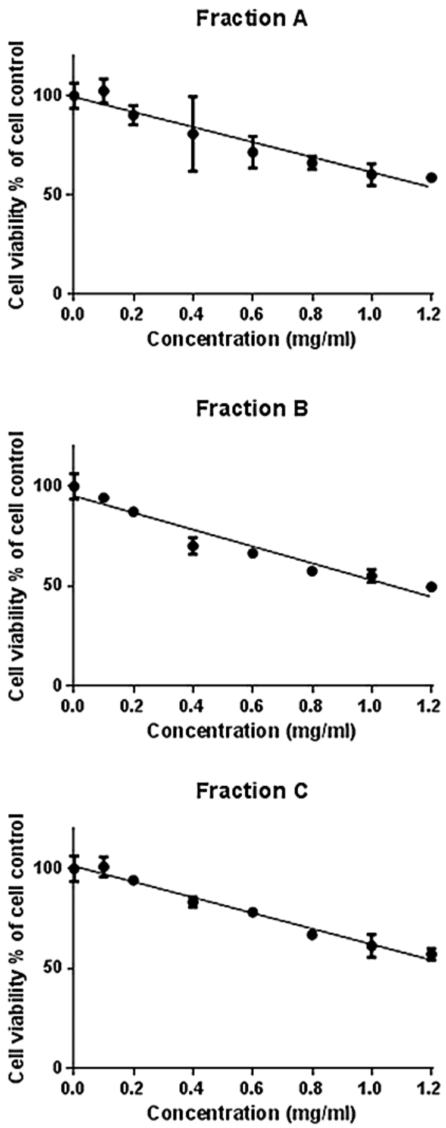


Figure 1. Cytotoxic activity of GP fractions (A, B, and C) on the viability of Vero cell line at 72 h treatment. Data are presented as percentage (%) of cell control

3.4.2. Effect of GP fractions A, B, and C on the replication of sensitive and resistant to ACV HSV-1 and HSV-2 strains by determining the CPE effect of the virus on infected MDCK cells

The effect of the studied fractions A, B, and C on HSV-1 and HSV-2 replication was investigated using the Vero cell line and the MTT test to detect cytopathic effects under viral influence. The test takes into account the survival rate of virus-infected cells treated with different concentrations of the extracts tested. It is mandatory to compare the survival of uninfected cells and those without the addition of substances (cell control) with that of infected cells without the addition of inhibitors (virus control).

Based on the data from the MTT test, the IC_{50} values of the studied fractions were determined. The 50% inhibitory concentration (IC_{50}) is the concentration of the test fraction that inhibits the virus-induced cytopathic effect by 50%. IC_{50} values indicate the antiviral efficacy of a substance, while SI provides information on its specificity and selectivity. The total IC_{50} and SI of the tested active fractions C and ACV, as determined by the MTT test, are presented in Tables 1 and 2.

Analysis of the antiviral results showed that fractions A and B did not exhibit suppression of viral replication. In contrast, the inhibitory effect of fraction C was selective, strain-specific, and dose-dependent. The hydroxybenzoic acids effectively inhibited the replication of the Victoria strain (HSV-1) at a concentration of 0.0001 mg/mL, equivalent to MNC (with 94.5% cell protection) (Table 1). In the present study, ACV, used as a positive control in the concentration range of 0.0005-0.05 mg/mL, was found to inhibit the replication of the HSV-1 strain Victoria (with 100% complete protection). In contrast, the *wt* HSV-2 strain Bja was 10 times less sensitive (Table 1).

The tested fraction C was twice as effective and selective as ACV in inhibiting the growth of ACV^R mutant DD (HSV-1), providing cell protection of 25.5%. On the other hand, the antiviral activity and selectivity of the same fraction against the ACV^R HSV-2 mutant (PU) and the *wt* HSV-2 strain Bja were very low. The IC_{50} is 0.1 to 0.03 mg/mL and 0.1 to 0.005 mg/mL, respectively. SI is 12 mg/mL. In comparison, ACV was neither an effective nor a selective inhibitor of the

Table 1. Antiviral activity of active fraction C against ACV-sensitive HSV strains (Victoria, HSV-1) and (Bja, HSV-2)

Tested agent	Antiviral activity					
	HSV-1 (strain Victoria)			HSV-2 (strain Bja)		
	% of protection of the cells in MNC	IC_{50} (mg/mL) ± SD	SI	% of protection of the cells in MNC	IC_{50} (mg/mL) ± SD	SI
Fraction C	94.5	0.01 ± 0.144	120	38.0	0.1 ± 0.005	12
ACV	100	0.0005 ± 0.02	800	95.7	0.005 ± 0.12	80

Table 2. Antiviral activity of active fraction C and ACV against HSV-1/HSV-2 ACV[R] mutants (DD, HSV-1) and (PU, HSV-2)

Tested agent	Antiviral activity					
	HSV-1 (strain DD)			HSV-2 (strain PU)		
	% of protection of the cells in MNC	IC_{50} (mg/mL) ± SD	SI	% of protection of the cells in MNC	IC_{50} (mg/mL) ± SD	SI
Fraction C	25.5	0.01 ± 0.004	21	10	0.1 ± 0.03	12
ACV	10.8	0.02 ± 0.05	20	0	0.02 ± 0.03	20

Table 3. Antibacterial activity of GP fractions

Bacterial strains	Minimal inhibitory concentration of the fractions			
	A [mg/mL]	B [mg/mL]	C [mg/mL]	Referent antibiotic* [mg/L]
Staphylococcus aureus ATCC 29213	> 5	> 5	*0.625*	P 0.25/G 0.25
Staphylococcus aureus ATCC 12 600	> 5	> 5	1.25	P 0.25
MRSA, *NBIMCC 8327*	> 5	> 5	1.25	G 0.25
Enterococcus faecalis ATCC 29212	5	> 5	1.25	P 2/G 8
Escherichia coli ATCC 25922	5	> 5	2.5	G 2
Pseudomonas aeruginosa ATCC 27853	> 5	> 5	1.25	G 0.5

Legend: *P = penicillin, G = gentamycin; A – Fatty acids and sterols fraction; B – Polar metabolites fraction; C – Phenolic compounds fraction

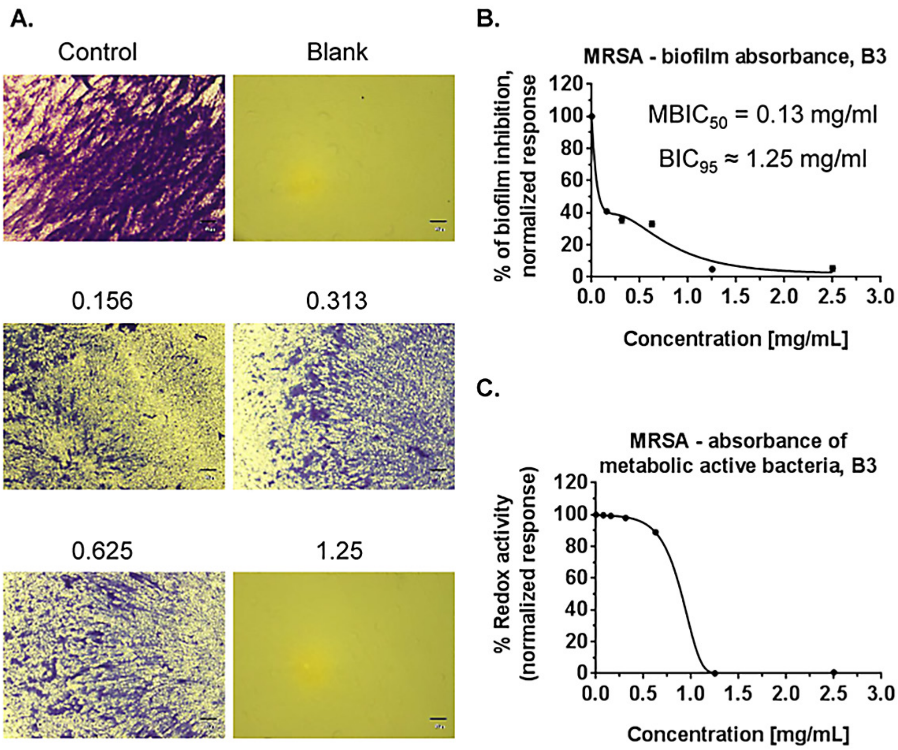


Figure 2. Inhibition of biofilm formation and metabolic activity of MRSA after 24 h incubation with fraction C obtained from *Graptopetalum paraguayense* E. Walter

A. Microscopic pictures representing the MRSA biofilm stained with crystal violet. All concentrations are in [mg/ml]; B. Crystal violet absorbance presented graphically with a dose-inhibition model; C. Absorbance of the MTT product formazan as quantitative parameter for the fraction of viable metabolically active bacterial cells.

Legend: $MBIC_{50}$ = median biofilm inhibitory concentration 50%; BIC_{90} = biofilm inhibitory concentration 90%

replication of the ACV[R] mutants DD (HSV-1) and PU (HSV-2), as confirmed by the IC_{50} values. Despite the low cellular protection provided by the phenolic compounds contained in GP, it is higher than that of acyclovir. When comparing the inhibitory ability of the phenolic fraction and the GP total extract, it can be concluded that, concerning wild-type HSV, both substances provide nearly the same cellular protection(Zaharieva et al., 2019). However, concerning ACV[R] mutants (strain DD), the total GP extract provided significantly higher cell protection than fraction C, 65.5% for the extract versus 25.5% for the fraction.

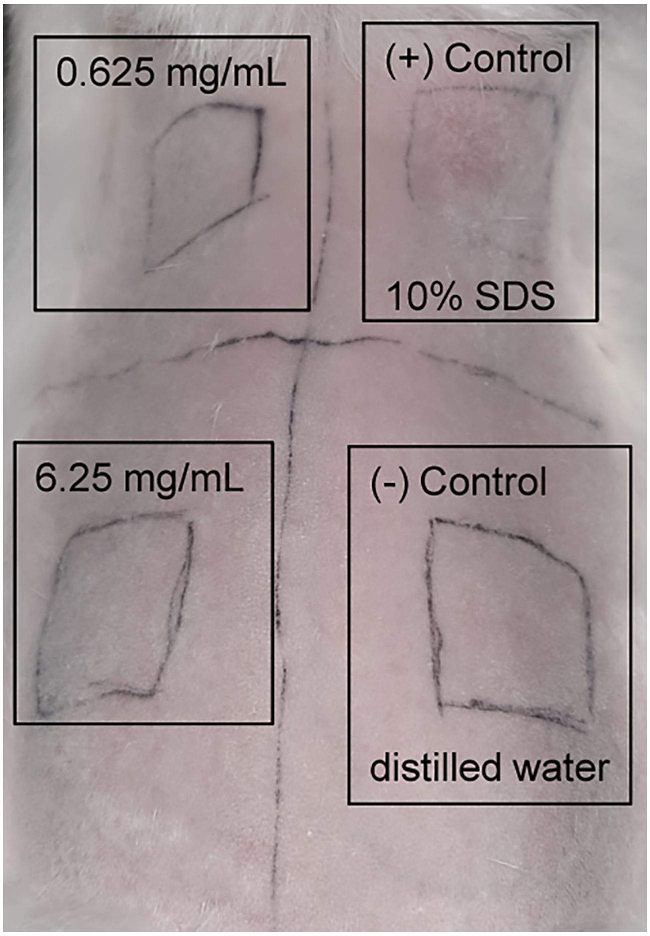


Fig. 3. Skin irritation test for phenolic compounds fraction from *Graptopetalum paraguayense* E. Walter

3.5. Inhibition of bacterial growth

Three different fractions obtained from *Graptopetalum paraguayense* E. Walter were tested for antibacterial activity using the BMD test to determine their MICs. The results from the test are presented in Table 3. The most active against all test strains was fraction C, with MICs two to eight times lower than those of the other two fractions. Let us compare the inhibitory activity of fraction C with that of the total extract of GP. It can be concluded that the phenolic compounds in the fraction inhibit bacterial growth to a greater extent (Zaharieva et al., 2019). The most sensitive strain to fraction C was *S. aureus ATCC 29213*, whose growth was inhibited by a concentration of C at 0.625 mg/mL, which is two- to fourfold lower than the MIC values determined for this extract on other bacterial strains. The most resistant strain was *E. coli ATCC 25922,* whose growth was inhibited at 2.5 mg/mL of the extract C. The other strains were equally affected by fraction C.

3.6. Inhibition of MRSA biofilm formation and redox activity

The capacity of fraction C to inhibit bacterial biofilm formation and redox activity was evaluated on the MRSA strain *NBIMCC 8327*. In Figure 2A, the results from the quantitative evaluation of the B3 effects on the MRSA biofilm formation are presented. A dose-dependent biofilm inhibition was observed after 24 h of exposure to C. As visible in the microscopic pictures, the extract eliminated almost the entire biofilm layer at a concentration of 1.25 mg/ml, which corresponds to the minimal inhibitory concentration for this strain. The other concentrations tested significantly inhibited biofilm formation compared to the untreated control. The quantitative evaluation of the data revealed an $MBIC_{50}$ of 0.13 mg/mL (Figure 2B). A concentration of 1.25 mg/mL eliminated approximately 95% of the biofilm layer (Figure 2B) and fully inhibited the redox activity of the remaining attached bacteria (Figure 2C).

3.7. Skin irritation in rabbits

As can be seen from the presented results on the antibacterial effect of hydroxybenzoic acids from GP, they mod-

Table 4. Primary irritation index

Time of exposure	0.625 mg/ml	6.25 mg/ml	10% SDS (positive control)
24 h (erythema + oedema)	0 (0/0)	0 (0/0)	6 (3/3)
PIS	0	0	3
PII	0	0	3

erately inhibit bacterial growth and MRSA biofilm formation. This means that the minimum inhibitory concentration of fraction C is high, and it can cause skin irritation when applied topically. Therefore, the skin irritation test aimed to evaluate the potential of the phenolic fraction from *Graptopetalum paraguayense* E. Walter leaves to induce skin irritation in albino rabbits and to establish criteria for analyzing and interpreting the results obtained. The results after 72 hours of exposure are presented in Figure 4 and Table 4. The fraction C of *Graptopetalum paraguayense* E. Walter did not induce in the treated animal any pathological alterations of the skin related to irritation, such as erythema or oedema, unlike the positive control. Since single exposure to C did not lead to skin irritation, the Cumulative Irritation Index (CII) was not calculated.

Conclusions

This study highlights the biological activities of *Graptopetalum paraguayense* E. Walter, showcasing its antiviral properties against the Herpes Simplex virus, as well as its antibacterial and anti-biofilm effects against MRSA. Three main fractions (A-polar metabolites, B-fatty acids, sterols, and tocopherols, and C-phenolic components) were extracted and analyzed using GC-MS, and then evaluated for anti-herpes simplex virus and antibacterial activity. The phenolic compounds effectively inhibited the replication of the acyclovir-sensitive HSV-1 strain Victoria at a concentration of 0.01 mg/mL, achieving 94.5% cell protection at the maximum non-toxic concentration. These phenolic compounds from GP provided limited cellular protection against acyclovir-resistant mutants DD (HSV-1) and PU (HSV-2), but the protection was greater than that offered by acyclovir. The hydroxybenzoic acids from *Graptopetalum paraguayense* exhibited moderate activity against Gram-positive bacterial strains, with *Staphylococcus aureus* being the most sensitive. Fraction C from GP did not cause any pathological skin reactions, such as erythema or edema, in treated animals.

4. Supplementary material

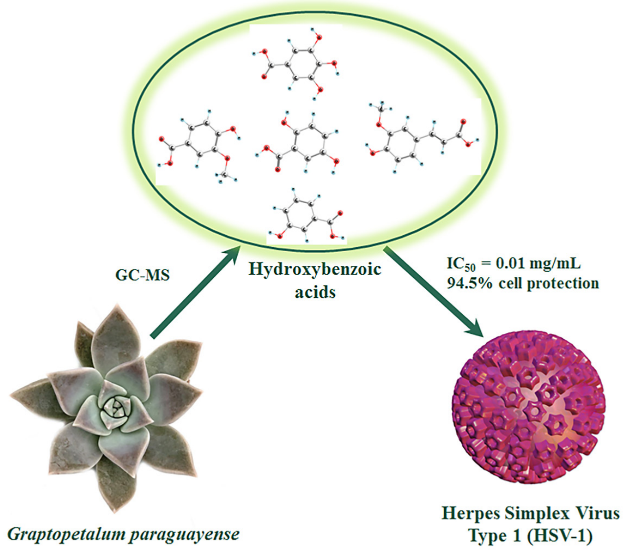
Additional supporting information may be found in the *Supplementary material* section at the end of the article:

Table S1. Components of fraction A (polar metabolites) from *G. paraguayense* determined by GC-MS analysis. DW – dried weight (μg/g), RT– retention time, RI – Kovàts retention indices, TMS – trimethylsilyl derivatives.

Table S2. Components of fraction B (fatty acids, sterols, tocopherols) from *G. paraguayense* determined by GC-MS analysis. DW – dried weight (μg/g), RT– retention time, RI – Kovàts retention indices, TMS – trimethylsilyl derivatives.

Table S3. Components of fraction C (phenolic compounds) from *G. paraguayense* determined by GC-MS analysis. DW – dried weight (μg/g), RT– retention time, RI – Kovàts retention indices, TMS – trimethylsilyl derivatives.

Figure S1. GC-MS chromatograms of the identified components from the GP fractions.



Graphical abstract

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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