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Evaluation of biological activities of goldmoss stonecrop (*Sedum acre* L.)

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Abstract: In vitro antioxidant, antimicrobial, and antiproliferative activities of methanol, acetone, and ethyl acetate extracts of *Sedum acre* L. were determined. Their total phenolic contents as well as the concentrations of flavonoids were also evaluated. The total phenolic content was determined with Folin-Ciocalteu reagent and it ranged between 18.25 to 181.75 mg gallic acid/g. The concentration of flavonoids in various extracts of S. *acre* was determined by using a spectrophotometric method with aluminum chloride and the obtained results varied from 8.42 to 173.42 mg rutin/g. Antioxidant activity was monitored spectrophotometrically and expressed in terms of IC_{50} (µg/mL), and its values ranged from 29.57 to 987.16 µg/mL. The highest phenolic content and capacity to neutralize DPPH radicals were found in the acetone extract. In vitro antimicrobial activity was investigated by the microdilution method. Minimum inhibitory concentration and minimum microbicidal concentration were determined. Testing was conducted against 17 microorganisms, including 13 strains of bacteria (standard and clinical strains) and 4 species of fungi. The tested extracts showed significant antibacterial activities against bacteria and weak to selective activities against the tested fungi. Antiproliferative activity of the methanol extract on the HCT-116 cell line was determined by MTT assay. Results showed that S. *acre* has medium activity on cell proliferation, with IC_{50} values of 281.69 for 24 h and 126.57 for 72 h. Based on these results, S. *acre* is a potential source of phenols as a natural antioxidant, antibacterial, and anticancer substance of high value. The phenolic content of extracts depends on the solvents used for extraction. The results of our study showed the great potential of S. *acre* for use in phytotherapy, pharmaceuticals, and the food industry.

Key words: *Sedum acre*, antimicrobial, antioxidant, antiproliferative activity

Introduction

Goldmoss stonecrop (*Sedum acre* L. - Crassulaceae) is a perennial herbaceous plant, up to 10 cm high, with smooth-margined succulent leaves 3-6 mm long and yellow flowers. It inhabits dry sandy soil, stones, and rocky surfaces in Europe, northern and western Asia, Anatolia, and North Africa (1). In traditional medicine it is widely used to treat ulcers and infected wounds, and as a hypotensive (2). The therapeutic activity of *S. acre* is mainly caused by alkaloids contained in this herb (3). Although it is mentioned in some papers dealing with wild medicinal herbs (3-7), there are no other data about the biological activities of the *S. acre* plant.

Nature has long been an important source of medicinal agents. An impressive number of modern drugs have been isolated or derived from natural sources, based on their use in traditional medicine (8,9). Evaluation of plant biological effects, such as antioxidative, antiallergic, antibiotic, hypoglycemic, and anticarcinogenic effects, makes a significant contribution to medicinal plant study as well as to their pharmaceutical applications (10,11).

Many medicinal plants have been proven important sources of phenolic compounds that show good antioxidant activity. Currently used synthetic antioxidants have been suspected to cause or promote negative health effects; hence, stronger restrictions have been placed on their application and there is also a trend of substituting them with naturally occurring antioxidants (12,13).

Plants are an abundant natural source of very effective antibiotic compounds. Plant antibiotics are chemicals that kill cells or inhibit their division and are classified into antibacterial, antifungal, antiviral, and antineoplastic types according to their target. They affect cell membrane permeability, and they regulate or disable enzymatic processes and protein translation (14,15).

A wide variety of secondary metabolites obtained from plants are tested for their ability to treat cancer (16). The relation between phenolic compounds and reduced cancer risk has been reported in previous studies that showed a decrease in cancer risk with consumption of vegetables and parts of plants rich in flavonoids (17).

The biological activity of *S. acre* is little known up to now. The aim of this study is to investigate the antioxidant, antimicrobial, and antiproliferative properties of the methanol, acetone, and ethyl acetate extracts of this plant, as well as to assess the biological activity in relation to phenol concentration and flavonoid content in the tested extracts.

Materials and methods

Chemicals

Organic solvents and sodium hydrogen carbonate were purchased from Zorka Pharma, Šabac, Serbia. Gallic acid, rutin hydrate, chlorogenic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent and aluminum chloride hexahydrate ($AlCl_3$) were purchased from Fluka Chemie AG, Buchs, Switzerland. Nutrient liquid medium, a Mueller-Hinton broth, was from Liofilchem, Roseto degli Abruzzi, Italy, while a Sabouraud dextrose broth was from Torlak, Belgrade, Serbia. An antibiotic, doxycycline, was purchased from Galenika A.D., Belgrade, and an antimycotic, fluconazole, was from Pfizer Inc., New York, NY, USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, Invitrogen, Carlsbad, CA, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA Laboratories, Pasching, Austria. Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from SERVA, Heidelberg, Germany. All other solvents and chemicals were of analytical grade.

Plant material

Aerial parts of *S. acre* were collected in June 2010 from natural populations in Trgovište in the area surrounding the city of Vranje in southern Serbia (42°23′14.27″N, 22°01′42.93″E; altitude: 574 m; exposition: W, habitat: arid, thermophilic, rocky). The voucher specimen of *S. acre* was confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at room temperature (20 °C).

Preparation of plant extracts

Prepared plant material (10 g) was transferred to dark-colored flasks with 200 mL of solvent (methanol, acetone, and ethyl acetate) respectively and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was reextracted with an equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C using a rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 °C.

Determination of total phenolic contents in the plant extracts

The total phenolic content was determined using a spectrophotometric method (18). The reaction mixture was prepared by mixing 0.5 mL of methanol solution (1 mg/mL) of extract with 2.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 mL of 7.5% $NaHCO_3$. The samples were incubated at 45 °C for 15 min. The absorbance was determined at λ_{max} = 765 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. A blank was concomitantly prepared with methanol instead of extract solution. The same procedure

was repeated for the gallic acid and the calibration line was construed. The total phenolic content was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of flavonoid concentrations in the plant extracts

The concentrations of flavonoids were determined using a spectrophotometric method (19). The sample contained 1 mL of methanol solution of the extract in the concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for 1 h at room temperature. The absorbance was determined at λ_{max} = 415 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for the rutin and the calibration line was construed. Concentration of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Evaluation of DPPH scavenging activity

The ability of the plant extract to scavenge DPPH free radicals was assessed using the method described by Tekao et al. (20), adopted with suitable modifications from Kumarasamy et al. (21). The stock solution of the plant extract was prepared in methanol to achieve a concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 μg/mL. Diluted solutions (1 mL each) were mixed with 1 mL of DPPH methanolic solution (80 μg/mL). After 30 min in darkness at room temperature (23 °C), the absorbance was recorded at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using the following equation: % inhibition = 100 × (A *control* – A *sample*) / A *control*). The IC_{50} values (inhibitory dose that inhibited cell growth by 50%) were estimated from the % inhibition versus concentration sigmoidal curve, using a nonlinear regression analysis. The data are presented as mean values ± standard deviation (SD) (n = 3).

In vitro antimicrobial assay

Test microorganisms

Antimicrobial activities of acetone, ethyl acetate, and methanol extracts were tested against 17 microorganisms, including 13 strains of bacteria (standard strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus pumilus* NCTC 8241; clinical strains: *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*, *Sarcina lutea*, *Salmonella enterica*, *Salmonella typhimurium*, *Sarcina lutea*, *Bacillus subtilis*, and *Bacillus cereus*) and 4 species of fungi: *Penicillium chrysogenum* PMFKG-F31, *Aspergillus niger* ATCC 16404, *Candida albicans* (clinical isolate), and *Candida albicans* ATCC 10231. All clinical isolates were a generous gift from the Institute of Public Health in Kragujevac. The other microorganisms were provided from the collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Bacterial and yeast suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparison with 0.5 McFarland's standard (22). The initial bacterial suspension contained about 10^8 colony forming units (CFU)/mL and a suspension of yeast contained 10^6 CFU/mL. Additionally, 1:100 dilutions of initial suspension were prepared into sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spores from slopes with growing aspergilli. The resulting suspensions were diluted to 1:1000 in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using the microdilution method with resazurin, an indicator of microbial growth (23). The 96-well plates were prepared by dispensing 100 μL of nutrient broth, Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi, into each well. A volume of 100 μL from the stock solution of tested extracts (concentration: 80 mg/mL) was added into the first row of the plate. Serial dilutions were then performed 2-fold by using a multichannel pipette. The obtained concentration range was from 0.0195 mg/mL to 40 mg/mL. MIC was defined as the lowest concentration of tested extracts that prevented a resazurin color change from blue to pink. The method was described in detail in a previous paper (24).

MMC was determined by plating 10 μL of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as the MMC.

Doxycycline and fluconazole, dissolved in nutrient liquid medium, were used as a positive control. The tested compounds were dissolved in DMSO and then diluted into nutrient liquid medium to achieve a concentration of 10% DMSO. A solvent control test was performed to study the effect of 10% DMSO on the growth of microorganisms. In the experiment, the concentration of DMSO was additionally decreased because of the 2-fold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Antiproliferative assay

Cell preparation and culturing

The HCT-116 cell line was obtained from the American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, with 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were grown in 75-cm^2 culture bottles supplied with 15 mL of DMEM, and after a few passages cells were seeded in 96-well plate. All studies were done with cells at 70% to 80% confluence.

Cell viability assay (MTT assay)

HCT-116 cells were seeded in a 96-well plate (10,000 cells per well). After 24 h of cell incubation, the medium was replaced with 100 μL of medium containing various doses of methanol extracts at different concentrations (1, 10, 50, 100, 250, and 500 μg/mL) for 24 h and 72 h. Untreated cells served as the control. After 24 h and 72 h of treatment, the cell viability was determined by MTT assay (25). The proliferation test was based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. At the end of the treatment period, MTT (final concentration: 5 mg/mL phosphate buffer solution) was added to each well, which were then incubated at 37 °C in 5% CO_2 for 2-4 h. The colored crystals of produced formazan were dissolved in 150 μL of DMSO. The absorbance was measured at 570 nm on a microplate reader. Cell proliferation was calculated as the ratio of absorbance of treated group divided by the absorbance of control group and multiplied by 100 to give percentage proliferation.

Statistical analysis

The data are expressed as the means ± SD. All statistical analyses were performed using SPSS 17 for Windows (SPSS Inc., Chicago, IL, USA). Mean differences were established by Student's t-test. Data were analyzed using one-way analysis of variance (ANOVA). In all cases, $P < 0.05$ was considered statistically significant.

Results and discussion

Total phenolic content, flavonoid concentrations, and in vitro antioxidant, antibacterial, and antifungal activities were determined with methanol, acetone, and ethyl acetate extracts of *S. acre*. Various solvents were used to achieve extraction of active substances with diversity in their polarity. This choice of solvents was proven to be very effective in earlier studies (26).

Total phenolic content

The content of total phenolic compounds in the examined plant extracts, which was determined by using Folin-Ciocalteu reagent, is expressed in terms of gallic acid equivalent (standard curve equation: $y = 7.026x - 0.0191$, $r^2 = 0.999$) as mg GA/g extract (Figure 1). The concentrations of total phenols in the examined extracts ranged from 18.25 mg/g to 181.75 mg/g. The high concentration of phenols was measured in the acetone and ethyl acetate extracts. Methanol extract had a considerably smaller concentration of phenolic compounds. The extracts obtained by using moderately polar solvents had higher concentrations of phenolic compounds. The content of phenols in extracts of *S. acre* depended on the polarity of the solvent used for extraction. High dissolubility of phenols in polar solvents provided a high concentration of these compounds in the extracts obtained by using polar solvents for the extraction (27,28).

Concentration of flavonoids

The concentration of flavonoids in various extracts of *S. acre* was determined by using a spectrophotometric

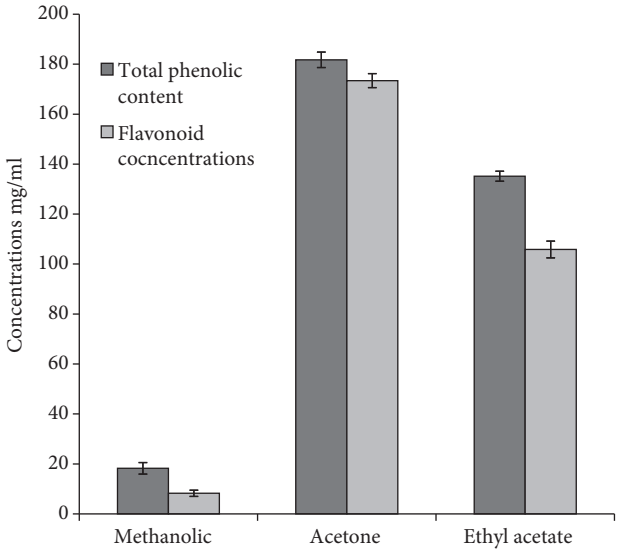

Figure 1. Total phenolic content expressed in terms of gallic acid equivalent (mg of GA/g of extract) and flavonoid concentrations expressed in terms of rutin equivalent (mg of RU/g of extract) in different *S. acre* extracts.

method using $AlCl_3$. The values for concentrations of flavonoids are expressed in terms of rutin equivalent (standard curve equation: y = 17.231x – 0.0591, r^2 = 0.999) as mg RU/g extract. A summary of the identified quantities of flavonoids in the tested extracts is shown in Figure 1. The concentration of flavonoids in plant extracts ranged from 8.42 mg/g to 173.42 mg/g. A high concentration of flavonoids was measured in the acetone and ethyl acetate extracts. The lowest flavonoid concentration was measured in the methanol extract. The concentration of flavonoids in plant extracts depended on the polarity of solvents used in the extract preparation (29). Based on the obtained values of the concentration of flavonoids in the examined extracts from *S. acre*, it was found that the highest concentration of these compounds was in the extracts obtained by using solvents of moderate polarity.

Antioxidant activity

The antioxidant activity of different plant extracts from *S. acre* was determined by using a methanolic solution of DPPH reagent. Unlike free radicals generated in vitro such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition, brought about by various additives. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into colorless products (i.e., 2,2-diphenyl-1-hydrazine or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm (30).

Table 1. Values[1] for antioxidant (DPPH scavenging) activity of *S. acre* extracts.

Extracts	IC_{50} (µg/mL)
Methanol	987.16 ± 1.69
Acetone	29.57 ± 1.21
Ethyl acetate	40.42 ± 1.05

[1]Average of 3 analyses ± SD.

The antioxidant activity of 3 different extracts from the whole plant of *S. acre* is expressed in terms of IC_{50} (µg/mL) values. A summary display of obtained IC_{50} values of the antioxidant activity of tested extracts is given in Table 1. Parallel to examination of the antioxidant activity of plant extracts, the values for 3 standard compounds (Table 2) were obtained and compared to the values of the antioxidant activity. The standard substances were rutin, chlorogenic acid, and 3-tert-butyl-4-hydroxyanisole (BHA). The obtained values of antioxidant activity examined with DPPH radical were in the range of 29.57 µg/mL to 987.16 µg/mL. The largest capacity to neutralize DPPH radicals was found for the acetone extract, which neutralized 50% of free radicals at the

Table 2. Values[1] of antioxidant (DPPH scavenging) activity of standard substances obtained for comparison with the values of *S. acre*.

Substances	IC_{50} (µg/mL)
BHA	5.39 ± 0.31
Rutin	9.28 ± 0.27
Chlorogenic acid	11.65 ± 0.52

[1]Average of 3 analyses ± SD.

Table 3. Antimicrobial activities of acetone, ethyl acetate, and methanol extracts of *S. acre*.

Species	Methanol extract		Acetone extract		Ethyl acetate extract		Doxycycline / Fluconazole	
	MIC*	MMC*	MIC	MMC	MIC	MMC	MIC	MMC
Escherichia coli ATCC 25922	2.5	20	2.5	5	2.5	10	15.625	31.25
Escherichia coli	5	40	2.5	10	20	20	7.81	15.625
Pseud. aeruginosa ATCC 27853	1.25	20	0.313	5	0.313	2.5	62.5	125
Proteus mirabilis	2.5	2.5	0.625	1.25	0.625	1.25	250	>250
Salmonella typhimurium	2.5	10	1.25	2.5	1.25	2.5	15.625	125
Salmonella enterica	2.5	5	1.25	2.5	–	–	15.625	31.25
Staphylococcus aureus	2.5	2.5	1.25	2.5	–	–	0.448	7.81
Staphylococcus aureus ATCC 25923	0.625	2.5	0.313	2.5	0.625	10	0.224	3.75
Sarcina lutea	0.039	2.5	0.039	2.5	–	–	<0.448	3.75
Bacillus subtilis	1.25	2.5	1.25	1.25	0.313	0.625	0.112	1.953
Bacillus cereus	0.039	0.625	0.039	0.625	–	–	0.977	7.81
Bacillus pumilus NCTC 8241	1.25	1.25	1.25	2.5	–	–	0.112	7.81
Candida albicans ATCC 10231	5	20	2.5	2.5	0.313	0.313	31.25	1000
Candida albicans	10	10	1.25	1.25	2.5	2.5	62.5	1000
Penicillium chrysogenum	10	10	10	10	10	10	62.5	500
Aspergillus niger ATCC 16404	10	20	10	20	20	20	62.5	62.5

*Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values are given as mg/mL for plant extract and μg/mL for antibiotics; –, not tested.

concentration of 29.57 μg/mL. A similar activity was found for the ethyl acetate extract. The lowest capacity to inhibit DPPH radicals was determined for the methanol extract.

In comparison to the IC_{50} values of BHA, rutin, and chlorogenic acid (Table 2), the acetone and ethyl acetate extracts of *S. acre* manifested a stronger capacity for neutralization of DPPH radicals.

Antimicrobial activity

The results of in vitro testing of the antimicrobial activities of acetone, ethyl acetate, and methanol extracts of *S. acre* are shown in Table 3. For comparison, the MIC and MMC values of doxycycline and fluconazole are also listed in Table 3. The solvent (10% DMSO) did not inhibit the growth of the tested microorganisms.

Antimicrobial activity of the tested extracts was evaluated by determining MICs and MMCs in relation to the 17 species of microorganisms. MIC and MMC values were in the range of 0.039 mg/mL to 40 mg/mL. Extracts showed selective antimicrobial properties while the activities depended both on the species of microorganism and on the type and concentration of extract. In general, the tested extracts demonstrated high antimicrobial activity, showing more potent inhibitory effects on the growth of bacteria than on fungi.

The weakest antimicrobial activity was shown by the methanol extract ($P < 0.05$). When the activity is observed according to groups of microorganisms, this extract was the only one with strong activity on gram-positive bacteria, but had the weakest activity on fungi ($P < 0.05$).

The tested extracts showed high antibacterial activity against gram-positive bacteria. MIC values were in the range of 0.039 mg/mL to 2.5 mg/mL. The extracts showed a significant effect in relation to *Bacillus cereus* (MIC: 0.039 mg/mL; MMC: 0.625 mg/mL) and *Sarcina lutea* (MIC: 0.039 mg/mL; MMC: 2.5 mg/mL). The extracts also showed good antibacterial effect on gram-negative bacteria. The best effect was with acetone and ethyl acetate extracts

on *P. aeruginosa* (MIC: 0.313 mg/mL; MMC: 2.5 and 5 mg/mL) and *P. mirabilis* (MIC: 0.625 mg/mL; MMC: 1.25 mg/mL).

The tested extracts showed low to moderate antifungal activity. MIC and MMC values were in the range of 1.25 mg/mL to 20 mg/mL. The exception was the ethyl acetate extract on the species *C. albicans* ATCC 10231, where the MIC and MMC were 0.313 mg/mL. These results are in accordance with the preliminary results of Tosun et al. (4) on the antifungal activity of the methanol extract of *S. acre* on *C. albicans* ATCC 10231 and *C. krusei* ATCC 6258.

While the acetate extract had good antifungal activity on *Aspergillus restrictus* as well as on *A. fumigatus* (6), the other extracts of *Sedum acre* had no significant activity on other filamentous fungi. The activity of the acetone extract was in relation with its total phenol and flavonoid content. In some previous studies it was claimed that the phenolic compounds from the plant might play a major role in their antimicrobial activities (31).

With the exception of the assay of anti-*Aspergillus* activities, this is the first study on the antimicrobial activity of the acetone and ethyl acetate extracts of *S. acre*. The results of our research indicated a good antimicrobial potential of *S. acre*, based on which it could be considered as a source of potential antimicrobial substances.

Antiproliferative activity

Secondary metabolites derived from natural sources have been receiving increasing attention in recent years since they were reported to have a remarkable spectrum of biological activities including antioxidant, antiinflammatory, antibacterial, and anticarcinogenic activities. They may have many health benefits and can be considered as possible chemopreventive agents against cancer (32,33).

In order to provide complete screening of biological activities, cell proliferation ability was also examined. To explore the antiproliferative activity of methanol extracts of *S. acre* on the HCT-116 cell line, the MTT cell viability assay was used. We treated HCT-116 cells with different concentrations of extract (in a concentration range from 1 µg/mL to 500 µg/mL) and determined cell viability after 24 h and 72 h.

The shape of dose-response curves indicated a significant inhibition of cell growth in a dose- and time-dependent manner (Figure 2). Cell growth was significantly lower ($P < 0.05$) when extract-treated cells were compared to control cells. The extract exhibited higher cytotoxic effects after a longer exposure. The effects of extracts were expressed by IC_{50} values. The IC_{50} value was used as a parameter for cytotoxicity. The IC_{50} value was 281.69 after 24 h and 126.57 after 72 h of exposure. The methanol extract of *S. acre* affects cell proliferation and inhibits cell growth, but according to the IC_{50} values, we can conclude that it has an intermediate effect on HCT-116 cell proliferation.

Results reported here contributed to the knowledge on the biological activity of *S. acre*. It could be concluded that *S. acre* is a source of phenolic compounds that demonstrate effective antioxidative, antibacterial, and antiproliferative activities. Acetone is a very effective solvent for extraction of phenolic compounds from this plant. The determined cytotoxic and antimicrobial activities of *S. acre* are of great importance in preservation of potential antimicrobial substances and they also present important information for further studies and applications in phytotherapy, pharmacy, and food industry.

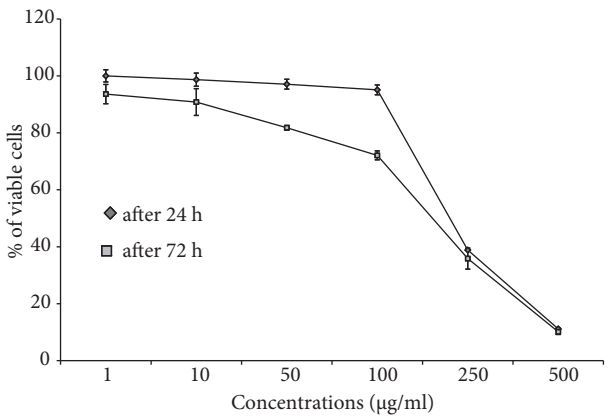

Figure 2. The dose-response curves of the effects of *S. acre* on cell growth in HCT-116 cells. The cells were treated with various concentrations of drugs after 24 h and 72 h of exposure. The antiproliferative effects were measured by MTT assay. Results were expressed as means ± standard error for 3 independent determinations.

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