PHYTOCHEMICAL AND ANTIPROLIFERATIVE POTENTIAL OF *HEDERA HELIX* EXTRACT FRACTIONS

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In our study a Hedera helix L. leaves extract was fractioned by flash chromatography. The extract and its nine fractions were tested for biocompatibility on NCTC normal fibroblasts, and antiproliferative activity on Hep-2 epithelial tumor cells. In vitro tests revealed that the samples were biocompatible on NCTC cells up to 200 μg/mL. Fractions 7 and 8 manifested strong antiproliferative effect on Hep-2 cells, and were analyzed versus ivy extract to determine the content in phytochemical constituents and antioxidant activity. The obtained results highlighted that the high content of saponins is responsible for the fractions 7 and 8 antiproliferative activity.

Keywords: *Hedera helix* L. extract; flash chromatography; extract fractions; antiproliferative activity; phytochemical constituents

1. Introduction

Hedera helix L. (Araliaceae), known as common ivy, is an evergreen climber woody plant found widespread throughout Europe, Asia and America. In traditional medicine *Hedera helix* was used for its analgesic, sedative, antiparasitic, cytotoxic and emmenagogue actions [1-3]. In present, ivy leaves and stems are used as multiple supplements, the plant being recorded in the European Pharmacopoeia as an herbal medicine due to its widely accepted effectiveness [4, 5]. Previously studies have shown that *Hedera helix* plant composition includes triterpenic saponins (hederacoside C, hederagenin, α hederin, oleanolic acid derivatives), flavonoids (rutin, quercetin), polyphenols (caffeic acid, derivatives of chlorogenic and rosmarinic acids), sterols (sitosterol and stigmasterol), sesquiterpenes and polyacetylenes (falcarinone and falcarinol), anthocyanins, coumarins, amino acids, vitamins, volatile and liquid oils, β-lecithines [5-7]. An important expectorant action of ivy infusion was demonstrated to be induced by the triterpenoid saponins active substances present in plant leaves [7-9]. Other

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scientific studies concerning the saponins from ivy, indicated that these compounds manifest strong cytotoxicity against various cancer cell lines and tumors *in vitro* and *in vivo* [8-11]. Quetin-Leclercq J., Elias et al. [8] studied *in vitro* cytotoxicity of ivy triterpenic saponins and revealed that most active compounds were α- and β-hederins. Other experiments of α-hederin on B16 mouse melanoma cells in serum-free media, induced apoptosis after only 8 h of treatment and inhibited the proliferation of 3T3 mouse normal fibroblast cells [9-11]. Also, Danloy et al. [12] in their study concluded that saponins cytotoxicity is reduced in the presence of Fetal Calf Serum (FCS) in the culture medium, suggesting that saponins form chemical bonds with proteins presented in the FCS.

In this paper a *Hedera helix* leaves hydroethanolic extract was fractioned by flash chromatography, followed by *in vitro* study of the ivy extract and its nine fractions, for evaluation of their biocompatibility on NCTC normal fibroblasts and antiproliferative activity on Hep-2 epithelial tumor cells. The fractions with strong antiproliferative activity were biochemically characterized versus ivy extract for determination of the phytocompounds amount and antioxidant activity in order to explain their *in vitro* biological activity.

2. Materials and Methods

2.1. *Hedera helix* L. leaves extraction

The commercial dried leaves of *Hedera helix* L. purchased from Hofigal SA, were identified with the specimen (voucher no. 407754) deposited at the Botanical Garden in Bucharest, Romania.

The extraction of dried *Hedera helix* L. leaves (100 grams) previously shredded, was performed by refluxing with ethanol/water 1:1 (500 mL) at 80ºC, with stirring for 2 h. ~~The ethanolic extract was filtrated and concentrated on a rotary evaporator~~.The hydroethanolic extract was filtered off and concentrated under vacuum using a rotary evaporator.

2.2. Flash chromatography fractionation of the ivy extract

The *Hedera helix* extract was fractionationed using flash chromatograph system (Biotage Isolera) with UV-Vis detector and autosampler Fraction Collector Rack Type 16x100mm.1 mL of ivy extract pre-absorbed onto a sample consisting in a support frit with silica gel, previously dried, was loaded for flash chromatography fractionation in a Biotage® SNAP Ultra C18 cartridge 10g, filled with solid phase of silica gel granules HP-Sphere^{TM} 10 µm. The mobile phase used was composed of *A)* acetonitrile and *B)* water with 0.1% phosphoric acid, at a flow rate of 10 mL/min and the scanning detection of 200–800 nm. The fractions were grouped by software according to the UV absorption spectrum, and nine fractions of approximately 10 mL, noted Fr.1 – Fr.9, were collected.

2.3. Cell culture tests

The cytotoxicity of *Hedera helix* samples was tested according to the international standard ISO 10993-5/2009. Biocompatibility test was performed on NCTC normal fibroblasts and antiproliferative activity was evaluated on Hep-2 epithelial tumor cells (ECACC Sigma Aldrich). Cells were cultivated in Minimum Essential Medium (MEM), supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine and a mixture of 100 U/mL penicillin, 100 μg/mL streptomycin and 500 μg/mL neomycin.

For the experiments, the cells were seeded in 96-well culture plates at a density of $4x10^4$ cells/mL for NCTC culture and $6x10^4$ cells/mL for Hep-2 culture, and incubated at 37°C, 5% CO_2, in humid atmosphere. After 24h of incubation, the samples were added at concentrations of 2, 5, 10, 25, 50, 100, 200, 300 and 400 μg/mL in NCTC culture in MEM supplemented with 10% FBS, while for Hep-2 tumor cell culture, a parallel testing was performed in two variants consisting in samples diluted in MEM with or without 10% FBS [12]. The samples were tested in triplicate and the culture plates were incubated for 24h. Cell viability was evaluated by MTT assay. The culture medium was replaced with 0.025 % (w/v) MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] in MEM and the cells were incubated at 37ºC for 3h [13, 14]. Then, the solution was replaced with isopropyl alcohol for dissolving the formazan crystals. The optical density (OD) was measured at 570 nm using a Berthold Mithras LB 940 microplate reader (Berthold Tehnologies, Germany). The result was calculated using the following formula:

$$\%\, cell\ viability = (OD\ sample / OD\ control) \times 100\ \% \quad (1)$$

where untreated cells were used as control. Control cells are considered to have 100% viability.

2.4. Phytochemical characterization

Total polyphenolic content was determined by Folin-Ciocalteu method. Samples of 0.15 mL were mixed with 0.75 mL Folin-Ciocalteu reagent (Fluka). After 5 min of incubation, 4 mL of 15% sodium carbonate solution were added and the the mixture was completed with water up to 15 mL. After 30 min of incubation at room temperature, the OD was read at 765 nm using an UV-VIS spectrophotometer (Jasco V-530, Japan). A standard curve was prepared using different concentrations of caffeic acid and was defined by the equation: y (conc. mg/L) = 0.0015 x + 0.0084, with a correlation coefficient of 0.993. Total phenolic content was expressed as mg caffeic acid/g dry weight of sample.

Total flavonoid content was determined using aluminium chloride colorimetric method. Samples of 0.5 mL were treated with 1.5 mL methanol, 0.1

mL solution 10% $AlCl_3$, 0.1 mL sodium acetate and 2.8 mL distilled water. The mixture was incubated for 30 min at room temperature. The OD of the reaction mixture was read at 415 nm in a microplate reader (Sunrise Tecan, Austria). Total flavonoid content in each sample was calculated from the calibration curve build using quercetin as standard [15-17]. The equation for the calibration curve was y=0.0038 x + 0.0064, with the correlation coefficient of 0.9993.

Antioxidant activity. A 7 mL ABTS ((2,2'-azino-bis-3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt) reagent was diluted to obtain an OD value of 0.7 (blank) measured at 734 nm using an UV-VIS spectrophotometer (Jasco V-650, Japan). Next, 1 mL ABTS was mixed with 0.1 mL test sample. After incubation at room temperature for 6 min. the mixture absorbance was read at 734 nm. A Trolox calibration curve was prepared in a concentration range of 0 – 250 μM [18]. The results were calculated as Trolox equivalents antioxidant capacity (TEAC), using the following formula:

$$TEAC\ sample = C\ Trolox \times f \times \frac{(OD\ sample - OD\ blank)}{(OD\ Trolox - OD\ blank)} \quad (2)$$

where: C_{Trolox} is Trolox concentration in mM/g dry weight and f is the sample dilution factor. The values were expressed as mM Trolox/g dry weight.

Total saponin content. Quantities of 1 mg from each sample were mixed in glass tubes with vanillin-acetic acid (0.2 mL, 5% w/v) and 0.8 mL of perchloric acid and heated to a temperature of 70°C for 15 min. The mixture was cooled on an ice bath, and then 5.0 mL of glacial acetic acid was added. The mixtures were measured on a UV-VIS spectrophotometer (Jasco V-650, Japan) at wavelength of 550 nm [19, 20]. Standard curve was prepared using different concentrations of ginsenoside Rb1. The equation was: y = 0.0102 x - 0.0191, with correlation coeficient: 0.999. Total saponins content of the samples was expressed as mg ginsenoside equivalent/g dry weight of sample.

4. Results and Discussions

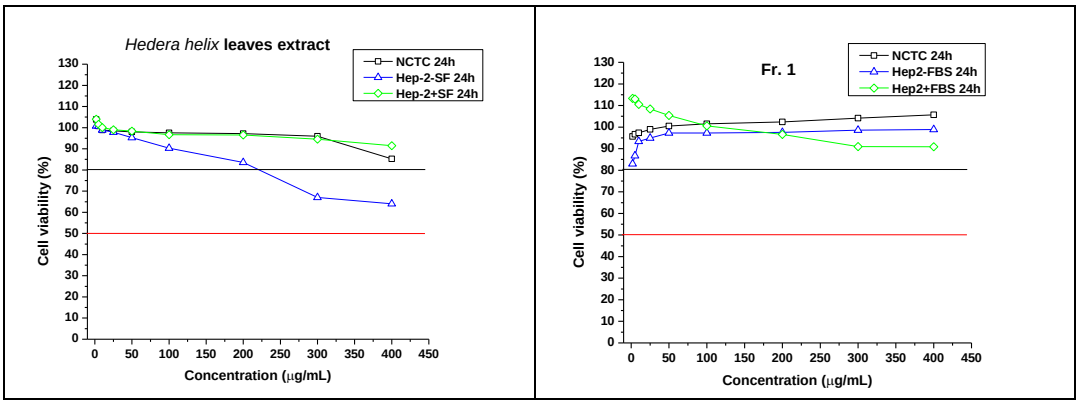
In our study 100g dried *Hedera helix* leaves were extracted by refluxing with 500 mL hydroethanolic solution, concentrated by reduced pressure to a syrup consistency extract (31 mL).

The ivy extract fractionation was performed by flash chromatography and nine extract fractions were obtained. The fractions in dry state were characterized by biological and biochemical methods.

Biocompatibility and antiproliferative evaluation of ivy extract and fractions

Hedera helix leaves extract and fractions have been tested *in vitro* on NCTC culture at 24 h, to determine their cytotoxicity. Our results showed that the extract and its fractions manifested biocompatibility on fibroblasts cells up to 200 µg/mL concentration. The extract stimulated cell proliferation (103.84% viability) at 2 µg/mL concentration. Also fraction 1 at 400 µg/mL and fraction 2 at 50 µg/mL induced good biocompatibility with viability results > 105%. The fraction 9 at 400 µg/mL registered highest biocompatible effect on normal cells with maximum of 114.57% cell viability, while fraction 8 at the same concentration induced severe cytotoxicity on normal fibroblasts with only 11.3% cell viability (Fig.1 and Fig. 2).

Antiproliferative activity testing of *Hedera helix* leaves extract and its nine fractions was performed on Hep-2 tumor cells, in variants of culture medium with or without fetal bovine serum [12] at 24h. All the samples tested at concentrations lower then 100 µg/mL in medium with or without FBS had no antiproliferative activity, with cell viability registered values >80%. The ivy extract and fraction 4 tested on Hep-2 tumor cells in MEM without FBS manifested a slightly antitumor activity in the concentration range of 300 - 400 µg/mL (64.04% viability). The antiproliferative evaluation of studied samples have revealed that only fractions 7 and 8 exhibited strong antiproliferative activity in the concentration range 200 – 400 µg/mL on Hep-2 cells in medium free of FBS, when values of tumor cell viability were of 1.98 - 44.2% for fraction 7, and 0.45 - 4.08% for fraction 8 (Fig.1 and Fig. 2).



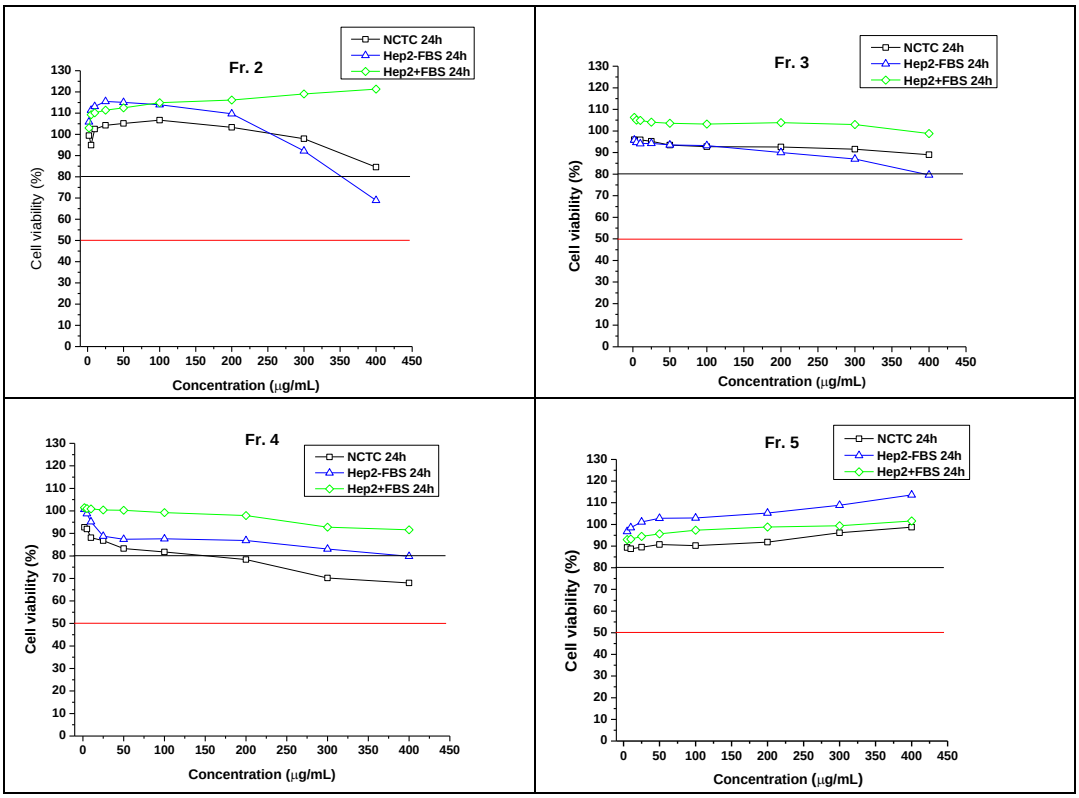
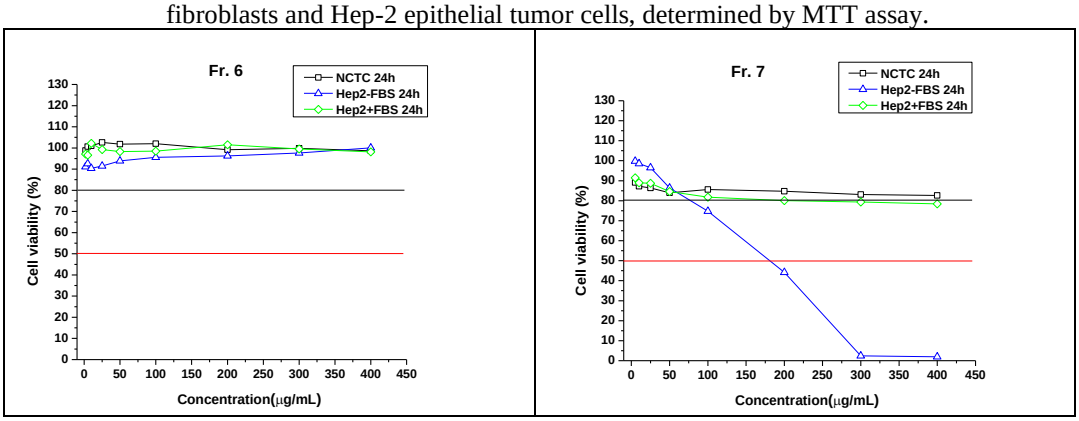


Fig. 1. Cell viability of ivy leaves extract and 5 of its fractions (Fr.1 – Fr.5) on NCTC normal fibroblasts and Hep-2 epithelial tumor cells, determined by MTT assay.



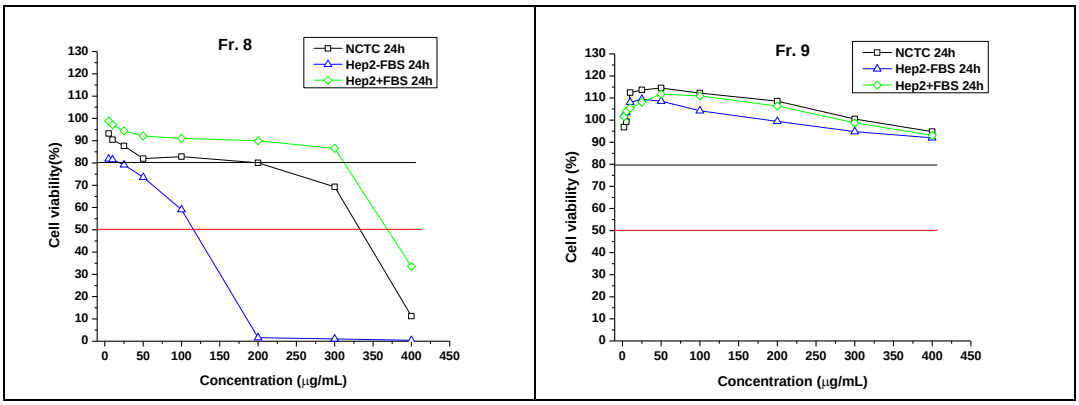


Fig. 2. Cell viability of ivy leaves extract fractions Fr. 6 – Fr. 9 on NCTC normal fibroblasts and Hep-2 epithelial tumor cells, determined by MTT assay.

The antiproliferative activity of samples was also expressed as the inhibitory concentration that killed 50% of cells (IC50, µg/mL) (Table 1).

Table 1

Values of IC50 determined for *Hedera helix* extract and its fractions on Hep-2 cell culture

Sample	IC50 (µg/mL)	
	Hep-2-FBS	Hep-2+FBS
Hedera helix extract	> 400	> 400
Fr.1	> 400	> 400
Fr.2	> 400	> 400
Fr.3	> 400	> 400
Fr.4	> 400	> 400
Fr.5	> 400	> 400
Fr.6	> 400	> 400
Fr.7	181	> 400
Fr.8	115.7	368.9
Fr.9	> 400	> 400

The fraction 7 registered an IC50 of 181 µg/mL in conditions of using culture medium free of FBS, while fraction 8 in the same conditions had an IC50 value of 115.7 µg/mL, respectively 368.9 µg/mL in medium with FBS.

Phytochemical characterization of ivy extract and fractions 7 and 8

The samples with strong antiproliferative effect, fractions 7 and 8 were biochemically evaluated versus ivy extract in order to reveal the correlation between the amount of phytocompounds such flavonoids, polyphenols and saponins and their antioxidant and antiproliferative activities.

Our data showed that the ivy extract and fraction 7 with rich content of polyphenols induced higher antioxidant activity compared to fraction 8 (Table 2). The antioxidant capacity measured by TEAC assay was higher in ivy extract and

fraction 7 (199.27 ± 4.44 and 59.37 ± 2.63 mM Trolox/g d.w., respectively), than the value registered for fraction 8 (22.11 ± 1.31 mM Trolox/g d.w.). Previous studies showed that several biological activities have been attributed to phenolic compounds, including anti-inflammatory, antioxidant and antiproliferative activities and apoptosis [21, 22].

The total saponin content was higher in the ivy extract (30.02 mg/g d.w.) then fraction 7 (10.30 mg/g d.w.) and fraction 8 (11.64 mg/g d.w.), however our *in vitro* studies on Hep-2 tumoral cells showed that fractions 7 and 8 with high content of saponins and small amount of phenolic respectively flavonoid content, manifested strong antiproliferative effect. The obtained results of our work are in good correlation with previous data on the evaluation of saponins antiproliferative activity [8 -12].

Table 2

Phytochemical characteristics of *H. helix* extract and fractions 7 and 8

Sample	Total phenolic content* (mg caffeic acid/ g d.w.)	Total flavonoid content* (mg quercetin/ g d.w.)	TEAC* (mM Trolox/ g d.w.)	Total saponin content*(mg ginsenoside/ g d.w.)
H. helix extract	44.02 ± 0.26	21.75 ± 0.51	199.27 ± 4.44	30.02 ± 0.68
Fr.7	11.60 ± 0.50	4.18 ± 0.14	59.37 ± 2.63	~~41.30 ± 0.26~~ 10.30 ± 0.26
Fr.8	8.03 ± 0.25	4.75 ± 0.21	22.11 ± 1.31	~~44.90 ± 0.19~~ 11.64 ± 0.19

*Each value was obtained by calculating average of three experiments ± standard deviation.

5. Conclusions

Flash chromatography can be used as an optimal method for fractionation of *Hedera helix* extract. We have obtained nine fractions of ivy extract by this method, which were noncytotoxic on fibroblasts cells up to 200 μg/mL concentration. Our results suggested that the antioxidant activity of ivy extract and its fractions were related to phenolic and flavonoid contents.

Antiproliferative activity of the studied samples, measured on human tumoral cervix cells was dependent on their saponin content. Fractions 7 and 8 from *Hedera helix* extract with high content of saponins manifested strong antiproliferative effect. Future studies must be performed in order to demonstrate the potential of the ivy extract fractions as agents for cancer therapy.

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