Selective Protective Effect of Antioxidant-rich *Rumex acetosa* Extracts

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Ethanolic extracts of Rumex acetosa leaves (Polygonaceae) are explored in terms of extraction methodology versus antioxidant capacity. Lyophilized and thermal-dried extracts at various alcohol concentrations are investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) bleaching method, the trolox equivalent antioxidant capacity (TEAC), the hemoglobin ascorbate peroxidase activity inhibition (HAPX) assay, total phenolic contents cf. the Folin-Ciocalteu method, as well as thin layer chromatography (TLC). The highest antioxidant content/activity is found for 60% alcohol. Cytotoxicity studies on this extract reveal a protective effect on the normal Hfl-1 as well as on HeLa tumoral cells against ionizing irradiation. Interestingly, a protective effect against the toxicity induced by the cytostatic drug, oxaliplatin, is seen in the healthy cells but not in the tumoral ones.

Keywords: antioxidant capacity, Rumex, cytotoxiciy, free radical; hemoglobin, HeLa

Many species of the Poligonaceae family have been studied for their biological activity, as alternative or complementary medicine for a various therapeutic purpose [1]. Some research are focused on the evaluation of the antibacterial and antioxidants activities of different plant parts such as *Rumex* species ((*R. vesicarius* [2], *R. japonicus* [3], *R. crispus* [4], *R. dentatus* [5], *R. patientia* [6] and *R. abyssinicus* [7]) while others have studied their cytotoxicity [8] (R. L [9] and *R. abyssinicus* [7]) and antimicrobial activities (*R. martimus* [10]).

Rumex acetosa (sorrel) is a perennial herb used in traditional medicine as anti-inflammatory, diuretic, antimicrobial and anticancer agent [11]. The aerial parts contain several flavonoids and polyphenols with antioxidant capacity, while its rhizomes are rich in antraquinones and polysaccharides presumably responsible for its antitumor effect [12]. The antiulcer property of *R. acetosa* in an animal model was recently demonstrated for the first time [11]. Other studies revealed that emodin, an antraquinone isolated from the aerial parts of *Rumex acetosa*, have cytotoxic and antimutagenic effects, which can be a subject of further studies for its medical application [12].

In the present study, the antioxidant activities of the alcoholic extracts of *Rumex acetosa* were determined with three different methods (Trolox equivalent assay, DPPH and hemoglobin ascorbate peroxidase activity inhibition), in conjunction with phenolic content and selective behavior in cytotoxic tests on normal and tumoral cell lines.

Experimental part

Materials and methods

Sorrel leaves were collected during the autumn period from the Sãlaj county in Romania. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH, and ascorbic acid were purchased from Sigma-Aldrich (Germany). The Folin-Ciocalteu reagent (standard solution 2 N, 1: 10 dilution) was obtained from Merck KGaA (Germany). Bovine hemoglobin was purified according to the Antonini and Brunori procedure [15], and was oxidized to met hemoglobin by ferricyanide treatment.

Silica gel $60F_{254}$ TLC aluminum plates (10x10cm) were purchased from Merck - Darmstadt (Germany). UV-Vis spectra were recorded on a Cary 50 UV-vis spectrometer (Varian). The cytotoxicity was performed on a BioTek synery 2 plate reader.

Sorrel leaves were dried using either heating at 40°C at atmospheric pressure, or lyophilization at -39°C under 0.13 mbar. Before lyophilization, leaves were frozen at -80°C, for 2 h. Extractions of the dried material were performed in 40, 60, 80 and 100% alcohol, respectively.

Thin layer chromatography

The TLC plates were developed in normal chromatographic twin trough chamber (Camag) using three different mobile phase for separation of flavonoids (ethyl acetate: formic acid: water - 18:1:1, v/v/v), of catechins (toluene: acetone: formic acid - 13:13:4, v/v/v) and of stilbenes (hexane: 2-propanol: acetic acid - 80:18:1:1, v/v/v/v). The detection was performed under UV light (254 nm and 366 nm) and under visible light and the documentation of the plates was performed using a TLC visualizer device (Digistore 2 - Camag).

The total polyphenolic content was measured in accordance with Folin-Ciocalteu assay descried in detail in [14]. For each sample, 1.5 mL Folin-Ciocalteu reagent (standard solution, 2N) were added to 300 μL sorrel extract 25 times diluted. The samples were incubated in the dark for 5 min. Then, 1.2 mL sodium carbonate (0.7 M) were added and samples were incubated in the dark for 2 h while the solution turned blue to different degree, depending the sample. The end-point absorbance at 760 nm of each sample was recorded on a Cary 50 UV-vis spectrometer (Varian).

Trolox equivalent antioxidant capacities (TEAC) assay

To measure the antioxidant activity kinetic measurements were performed. Thus, in a glass cuvette,

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2 μL sorrel extract (diluted 5 times) were added to the ABTS solution (formed by 900 μL PBS and 5 μL of 95 mM ABTS•$^+$ enzymatically prepared by incubation of ABTS with horseradish peroxidase and peroxide). The extracts were diluted with the same solution used for extraction (100, 80, 60, 40% alcohol). The process was monitored at 420 nm, for 600 s. Typical decay curves were obtained for every sample, allowing one to calculate the total change in absorbance (ΔA), which correlates directly to the antioxidant capacities of the samples, as well as the calculation of the area under the curve, which is inverse correlated with the antioxidant capacities.

Separately, ABTS•$^+$ consumption was also monitored by electron paramagnetic resonance (EPR) spectroscopy; these data were similar to the ones recorded in UV-vis at 420 nm, and are therefore not shown.

DPPH assay

A stock solution of 0.09 mg/ml DPPH was prepared by dissolving DPPH in methanol. To measure the antioxidant capacity, 4 μL sorrel extract were added to the 700 μL PBS + 250 μL DPPH, in a glass cuvette. The process was monitored spectrophotometrically at 517 nm, for 600 s, at room temperature. The same parameters, as in the TEAC assay, were calculated (ΔA and area under the curve).

Inhibition of hemoglobin ascorbate peroxidase activity (HAPX) assay was performed as in [13, 14]. Thus, in a quartz cuvette, 7 μL ascorbate of 50 mM and 15 μL H_2O_2 of 63 mM were added to 950 μL sodium acetate buffer, *p*H 5.5. Absorbance changes were monitored at 290 nm, at room temperature. After absorbance stabilization, the reaction was started by the addition of 8 μL met-Hb of 4.22 mM, followed by the addition of extract. The values given in table 1 represent the ratio between the slope obtained in the presence of hemoglobin + extract and the slope obtained only in the presence of hemoglobin. A high value is correlated with a good inhibition of hemoglobin ascorbate peroxidase activity as well as with a good antioxidant capacity.

Cytotoxicity.

Cytotoxicity was determined using a direct colorimetric assay adapted on the Mossman MTT method [16], after treatment of the normal immortalized human cell line HF-1 and human tumoral cell line HeLa with sorrel extracts. This assay is based on the enzymatic properties of living cell to transform the MTT dye tetrazolium ring into a purple-colored formazan structure. The color intensity is directly proportional with the number of viable cells and can be mathematically quantified.

The 96 wells containing the cell culture were incubated for 24 h under sterile conditions. Then, the microplates were treated with sorrel extract. Three independent experiments were performed for every cell line. In the first experiment, the samples were treated only with natural extract. In the second experiment, the samples were irradiated with a Co source used in therapeutic purposes (Theratron 1000) with 2 Grey, at 3 h after the application of extract. In the third case, 5 μM of oxaliplatin were added to each sample, 3 h after extract treatment. Absorbance measurements were made after 24 h of incubation on a BioTek Synery 2 plate reader. The results were analyzed using the Graph Pad Prism 5 biostatistics program.

Results and discussions

The effect of drying methods as well as of different concentrations of alcohol upon the extraction process was analyzed through some analytical and biochemical methods, in order to establish the best condition for a good extraction with a high antioxidant activity.

Cromatographic analyses

After chromatographic separation of extracts using the proper mobile phase, a series of antioxidants from the flavonoid and stilbenes/stilbenoids classes were observed. The chromatogram shown in figure 1 indicates that the best extraction of stilbenes occurs at 100% alcohol, regardless the drying conditions. For flavonoids and for their subclass, catechins, it is difficult to establish the best condition of the extractions (data not shown).

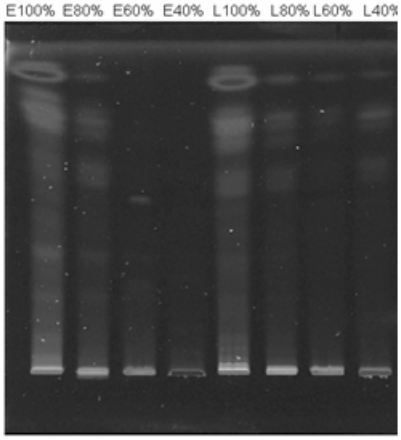

Fig. 1. TLC separation of the *Rumex acestosa* extracts on silica gel 60 F_{254} with hexane: 2-propanol: acetic acid (80:18:1:1, v/v/v/v) as mobile phase. Visualization in UV light at 366 nm. The labels are indicative for the thermal-dried (E) and lyophilized extracts (L) at various alcohol concentrations (100, 80, 60 and 40 %).

UV-vis spectra

Most antioxidants contain conjugated systems of double bonds arranged in either linear chains or aromatic systems, which absorb light in the UV-vis range. Three absorption maxima are seen in the both lyophilized and thermal drying samples (fig. 2), at 260, 330 and 675 nm. At 260 and 330 nm the highest intensity of the spectra was observed for the extracts containing 60 and 80% alcohol; one would thus expect a significant increase of the antioxidant capacity of these samples compared to the others (with 40 and 100 % alcohol). At 675 nm, as well as at 440 and 500-550, in regions characteristic for photosynthetic pigments (chlorophyll), the intensity of the spectra are correlated with the alcoholic concentration of sample.

Comparing the spectra at the same alcoholic concentration (data not shown) it is found that for 40 and 100% alcohol there are significant differences of the absorption bands and thus in the contents of the samples obtained via the two drying methods – as an illustration for the extent to which the thermal process can alter the organic material through partial oxidation and condensation.

Determination of total phenolic content

The total phenolic contents of the studied extracts were determined spectrophotometrically according to Folin-Ciocalteu procedure. As shown in figure 3, the results, given in gallic acid equivalents (GAE), vary from 8.70 to 54.81 mg/mL. The highest phenol content was recorded for E 60%, while E 100% contained the lowest amount of total phenols. There appears to be a general agreement with the intensity of absorptions seen in the UV-Vis spectra in figure 2, at e.g. 330 nm. Thus, the lowest absorbances in

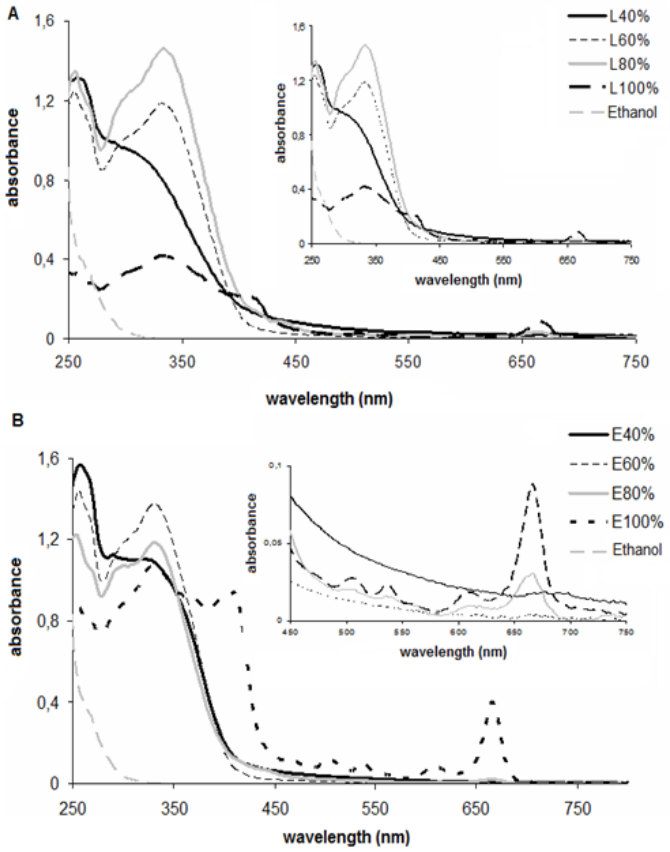

Fig. 2. UV-vis spectra of A) lyophilized (L) and B) thermal-dried extracts (E), diluted 40 times, at different concentration of alcohol (40, 60, 80 and 100 % alcohol)

figure 2 are for the 100% alcoholic extracts – which are also seen to have the lowest polyphenolic contents; moreover, the distinctly larger absorbance of the lyophilized 100% extract is paralleled by a distinctly higher polyphenolic content compared to the thermally-dried 100% extract. For the E samples the entire series of trends is identical between figures 2 and 3, while for the L samples the order of the 40% and 60% extracts is slightly reversed.

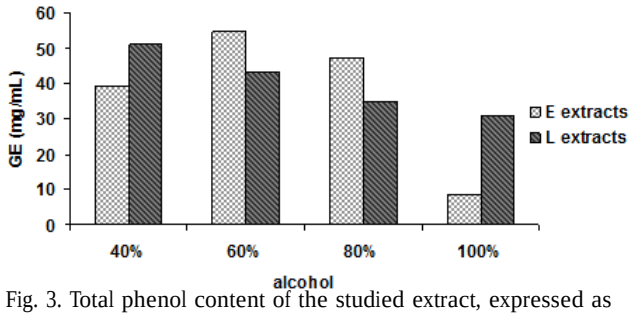

Fig. 3. Total phenol content of the studied extract, expressed as gallic acid equivalent (GAE). The labels E and L extracts are indicative for the thermal-dried and lyophilized extracts, respectively

Antioxidant activity

The antioxidant activity was evaluated using tree different method: ABTS, DPPH and HAPX. The results obtained, given in table 1, show a good (but not perfect) correlation between ABTS and DPPH, but a weak correlation with Folin-Ciocalteu. The latter may be due to the presence of other antioxidants in the sample, which are undetectable with Folin-Ciocalteu reagent, such as vitamin E or uric acid. There is a tendency of decreasing the antioxidant capacity with the increase in the alcoholic concentration used for extraction. Therefore, according to the ABTS and DPPH results, the best antioxidant capacity is seen for 40 and 60 % alcohol. Also, the ABTS results reveal a better capacity to quench free radicals for the E samples (exception E100).

Interestingly, an inverse correlation can be observed between ABTS/DPPH data and the inhibition of hemoglobin ascorbate peroxidase activity (HAPX). This method is based on the capacity of the antioxidant(s) to interact with the highly oxidant species (ferryl, globin radicals) generated during the catalytic cycles of the reaction between hemoglobin and peroxides [13,14].

However, if the values obtained with all three assays are normalized by dividing to the absorbance at 330 nm (a maximum present in all samples and arguably indicative of the general content in organic compounds regardless of their antioxidant activity), the extracts containing100% alcohol appear to have the best normalized antioxidant activity - most probably due to the fact that the alcohol-soluble antioxidants are, per number of aromatic/ unsaturated functional groups, stronger than the water-soluble ones (data not shown).

Cytotoxic activity

A cytotoxic assay was performed against two types of cell: normal Hfl-1 and tumoral HeLa cell, using one of the extracts seen to have the most efficient antioxidant activity, namely E60. The sorrel extract did not display any toxic effect on the normal HFl-1 cells; on the contrary, an increase in the cell proliferation could be observed (fig. 4). Irradiation at 2 Gy, as well as oxaliplatin treatment, are known to decrease cell viability in normal as well as tumoral cells. Perhaps expectedly, a positive effect of the sorrel extract is seen on the cell proliferation. The normal cell survival increase statistical significantly in comparison with the untreated control (nonparametric test t, Wilcoxon post-test, confidence interval 95%), but in the absence of a

	ABTS		DPPH		HAPX
	Area	ΔA	Area	ΔA	Slope ratio
E100%	460 ± 0	0.08±0.00	498±4	0.060±0.004	2.50±0.10
E80%	368 ± 8	0.16±0.02	490±2	0.136±0.005	1.96±0.04
E60%	326 ± 15	0.22±0.01	494±0.8	0.143±0.003	1.22±0.01
E40%	317 ± 2	0.23±0.01	495±6	0.124±0.003	1.13±0.02
L100%	429 ± 7	0.13±0.00	514±2	0.079±0.001	2.04±0.01
L80%	395± 11	0.16±0.00	497±3	0.122±0.005	1.72±0.06
L60%	355± 10	0.19±0.01	475±0.1	0.149±0.001	1.36±0.06
L40%	349± 9	0.18±0.01	488±12	0.138±0.001	1.17±0.02

Table 1
ANTIOXIDANT CAPACITIES OF THE STUDIED EXTRACTS ESTABLISHED BY ABTS, DPPH AND HAPX METHODS. AREAS ARE CALCULATED AS ABSORBANCE UNITS TIMES MIN

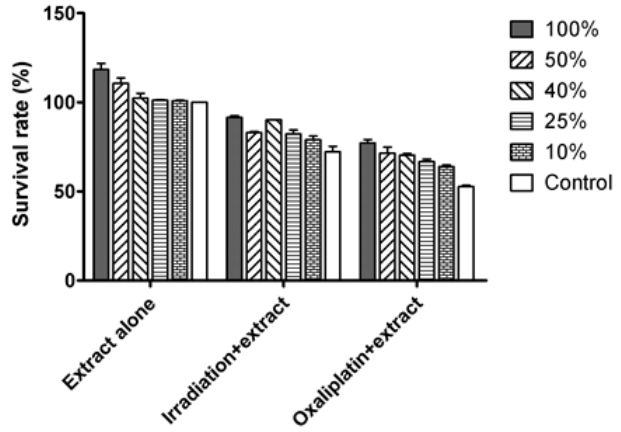

Fig. 4. The sorrel extract effect on normal Hfl-1 cells. The survival rate of the cell population subjected to different concentrations of herbal extract was studied in the presence or absence of sublethal irradiation and oxaliplatin treatment

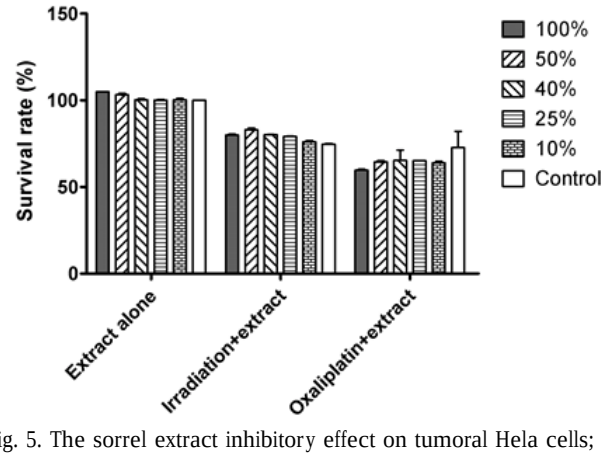

Fig. 5. The sorrel extract inhibitory effect on tumoral Hela cells; the impact of extract combination with irradiation or oxaliplatin treatment *in vitro*

dose-effect relationship. Irradiation at 2 Gy, as well as oxaliplatin treatment, are known to decrease cell viability in normal as well as tumoral cells. The irradiated and oxaliplatin-treated groups are significantly different from the group of cells treated with the extract alone (two-way ANOVA, column factor extremely significant $p<0.001$, row factor and interaction significant, $p<0.1$). The survival of the cells is obviously diminished when irradiated or treated with the chemotherapy drug, the trend being identical for all the concentration used (one-way Anova analysis, $p<0.5$). Conversely, in both irradiated and platinum-treated groups, the biggest sorrel concentration protects significantly the cells against the growth inhibitory effect of the treatments (one-way analysis of variance, Bonferroni multiple comparison of extract and oxaliplatin *versus* oxaliplatin alone, $p<0.5$). In cells treated with oxaliplatin, all sorrel concentrations, except the 10%, are capable to increase the cells survival.

In the case of HeLa cells (fig. 5) no dose-response relationship was observed and the plant extract does not display any statistically significantly effect (one-way ANOVA, Bonferroni post-test, in the 95% confidence interval). As well, within the irradiated and the platinum-treated groups the effect produced by different concentrations was inconsequential. But remarkably, when the plant extract was applied concomitant to the irradiation, all concentrations increased very significantly the growth inhibitory effect in comparison with their non-irradiated analogues (one-way Anova, all p values under 0.01). The oxaliplatin effect was also enhanced by the simultaneously sorrel treatment extract ($p<0.01$), except the 40% concentration, the phenomenon being understandable due to the lack of dose-response effect observed when the cells were treated with the extract only. Considering that the untreated cell population's survival was not significantly different following irradiation with the single dose or 5 μM oxaliplatin treatment (p value 0.0636, r squared 0.8407), we can conclude that the herbal extract increased the standard antitumor procedures effect on tumor cells.

Moreover, while the irradiation of HeLa cells leads to the same effect observed for the normal cells, the protective role of extract is considerably diminished. The cells treated with the antitumoral drug oxaliplatin show an even stronger difference: while Hfl-1 were stimulated by addition of the sorrel extract in a dose-dependent manner, HeLa remain clearly inhibited (fig. 5).

Thus, the *R. acetosa* extract does not have significant cytotoxic effect on the normal Hfl-1 and HeLa tumoral cell, but it displays a selective protection on normal cell viability against ionizing irradiation, and a slightly protective effect against the action of the cytostatic based platinum drug, oxaliplatin. This phenomenon is, remarkably, selectively manifested regarding normal cells, in contrast to the tumoral cells; this may lead to well-defined therapeutic applications of the extract as selective protective adjuvant designed to limit side-effects in chemotherapy.

Conclusions

It can be concluded from the results of the present study that all ethanolic extracts of sorrel leaves showed certain levels of antioxidant activity. A highest activity, paralleled by Folin-Ciocalteu data and UV-vis absorbances, was observed for extract containing 60% alcohol, obtained after thermal drying of the plant. Furthermore, the extracts did not display a cytotoxic effect, but interestingly, demonstrated a significantly protective effect on the normal Hfl-1 and HeLa tumoral cell against ionizing irradiation and a slightly protective effect against the toxic effect induced by the cytostatic drug, oxaliplatin; importantly, the latter effect is selectively seen in healthy but not in tumoral cells. Thus, these findings may lead to a therapeutic application of the extract to protect cells against the side effects induced by chemotherapy.

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