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## PROTECTION OF HIPPOCAMPAL AND ISLET BETA CELLS *IN-VITRO* BY EMODIN FROM LEAVES OF *RUMEX CONFERTUS*

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**ABSTRACT:** The natural anthracene derivative emodin (1,3,8-trihydroxy-6-methylanthraquinone) is known as an anticancer agent. In the present work, it was isolated for the first time from the ethanol extract of *Rumex confertus* leaves, widely used in Armenia as a vegetable. It was purified in two-step low-pressure liquid chromatography – a significantly easier and inexpensive procedure compared with those used for emodin purification. The purified preparation was characterized by chemical and TLC analyses, NMR, UV-Vis and fluorescence spectra. Rather high cytotoxicity toward cultivated primary cells of mice Ehrlich ascites carcinoma was demonstrated for both the ethanol extract ( $IC_{50} = 0.3 \pm 0.04 \mu\text{g/ml}$ ), and emodin ( $IC_{50} = 40 \pm 10 \text{ ng/ml}$ ) from *Rumex confertus* leaves. Along with the anticancer activity, these preparations protected a) hippocampal cells against toxic action of aggregated amyloid A $\beta$  (1-40) and A $\beta$  (1-42) peptides and b) the islet  $\beta$ -cells against death in the presence of aggregated pancreas peptide hormone amylin. The obtained results provide a rationale for developing anticancer, neuroprotective and antidiabetic remedies from the leaves of *Rumex confertus*.

**INTRODUCTION:** Emodin is a naturally occurring anthraquinone present in numerous plants, molds, and lichens. It is known mainly as an anticancer agent, exhibiting cytotoxic effects on many types of human cancer cell lines with the low  $IC_{50}$  values. Emodin induces apoptosis, suppresses angiogenesis, impedes metastasis, exerts an antiproliferative effect and inhibits the modulation of the cell cycle in specific oncogene over-expressed cells <sup>1, 2, 3</sup>. Its role in combination with standard chemotherapy drugs to reduce their toxicity and to enhance efficacy is investigated vigorously.

For example, emodin is able to potentiate the antitumor effect of gemcitabine, the standard first-line chemotherapeutic agent for a highly aggressive malignant disease on pancreatic cancer via promotion of apoptosis <sup>4</sup>. From the herb *R. acetosa* several anthraquinones were isolated. They were all provided with high cytotoxicity against 5 human tumor cell lines, and emodin showed the best toxicity <sup>5</sup>.

Several other therapeutic significance of emodin is under attention too. The pharmacological effects of emodin had been evaluated for anti-neoplastic, anti-inflammatory, anti-angiogenesis, antibacterial, anti-*Herpes simplex virus*, etc. activities <sup>6, 7, 8, 9, 10, 11</sup>. Along with these effects, emodin, extracted from the traditional Chinese medicinal herb *Polygonum cuspidatum*, protected the cultured cortical neurons against aggregated A $\beta$  (25–35) toxicity via inhibition of peptide-induced apoptosis <sup>12</sup>. Based

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on the fact that emodin regulates glucose homeostasis, it was suggested that it may serve as a therapeutic principle in the treatment of type 2 diabetes<sup>13</sup>. A potential role of emodin in the treatment of diabetic nephropathy has been shown<sup>14,15,16</sup>.

Armenia has a rich history in the field of phytotherapy<sup>17</sup>. However, at present, little information is available about the anticancer and other therapeutic properties of several dietary plants widespread in Armenia and used in folk medicine. In our previous work<sup>18</sup>, the ethanol extracts of leaves of grape, sorrel and sea buckthorn, rose petals, melilot, the membrane of walnut kernels were screened for their ability to suppress the growth of cultured primary cells of mice Ehrlich ascites carcinoma (EAC). The rather low IC<sub>50</sub> values in the inhibition of EAC cells' growth were evaluated for the extracts from rose petals, leaves of grape and sorrel (*Rumex crispus*). Based on the obtained results we presumed that these extracts and some their constituents could be recommended as sources for cancer prevention therapeutics.

In Armenian cuisine, *Rumex genius* is widely used since ancient times<sup>19</sup>. Although several plants have potentials for therapeutic use, the questions that remain to be answered are which components of these dietary plants are responsible for the observed effects and which is their molecular mechanism of action. In the present study, for the first time, one of the anthracene derivatives (AD) was purified from the ethanol extract of leaves of *Rumex confertus* using two steps of low-pressure liquid chromatography. On the bases of chemical and TLC analyses, NMR, UV-Vis and fluorescence spectra, it was identified as emodin. The anticancer activities of the ethanol extract and emodin from *Rumex confertus* were corroborated. Besides, their ability to protect the cultured hippocampal cells and pancreas islet  $\beta$ -cells from the toxic actions of aggregated amyloid A $\beta$  (1-40) and A $\beta$  (1-42) peptides, and the pancreas peptide hormone amylin, respectively, was shown for the first time.

#### MATERIALS AND METHODS:

**General:** Cell cultivation RPMI-1640 medium, supplements and cyclophosphamide monohydrate (CPA), were purchased from Sigma Ltd, USA; A $\beta$

(1-40/42) peptides – from China Peptide, China; amylin – from Gene Cust, Luxemburg; G-25 and LH-20 Sephadex – from Pharmacia Biotech, Upsala, Sweden. All the other chemicals and solvents were of the highest purity.

Spectral measurements were performed on Specord M-40 UV-VIS spectrophotometer (Germany) and spectrofluorometer Perkin-Elmer MPF-44A (USA), using quartz cuvettes with light path 0.5 and 1cm at 25°C in thermostatic cuvette holders. NMR spectra were recorded in CD<sub>3</sub>OD on a Varian Mercury Plus 400 spectrometer at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C. Chemical shifts ( $\delta$ ) in ppm units concerning the residual solvent signal and the coupling constant (J) in Hz are used.

**Plant Material:** The leaves of *Rumex confertus*, common name sorrel, were collected from the flanks of Mount Aragats in Armenia and dried in the shade. A voucher specimen has been deposited at the herbarium of the Botanical Department of Yerevan State University (Dr. Narine Zaqaryan). The dried material was ground, 10% (w/v) extract was prepared in 70% ethanol (72 h at ambient temperature) and filtered. The chemical constituents of the extract were identified using the specific reactions as described in our previous work<sup>18</sup>. Then, the ethanol extract was dried at 37 °C and stored at -18 °C until use. The yield of the dried extract was 0.2 g per 1g of dried sorrel leaves (SL).

**Separation of Anthracene Derivative:** To fractionate the SL extract, 1-3 g was dissolved in 5-10 mL of 70% ethanol and subjected to gel-filtration on LH-20 Sephadex containing column (2.6 x 20 cm), equilibrated with 20% ethanol. The column was washed with stepwise increasing concentrations of ethanol. The eluted with 70% ethanol fraction, rich with anthracene derivatives (AD), was subjected to gel-filtration on G-25 Sephadex column (1.5 x 20 cm), equilibrated with 20% ethanol. After washing with 20% and 40% ethanol, the eluted with 70% ethanol fraction was collected and dried by evaporation at 37 °C. For future study, a portion of this fraction was weighed, dissolved in 70% ethanol and diluted in 20% ethanol to appropriate concentration.

**Thin-Layer Chromatography:** The SL extract and the isolated from it AD fraction was

characterized by thin-layer chromatography (TLC) analysis on silica gel sheets (silica gel, glass support, Fluke) in two different solvent systems, appropriate for ADs: ethyl acetate/methanol/water 100:17:13 and ethyl acetate/n-hexane 3:7. TLC pictures were revealed by 5% KOH in methanol.

**Primary Cell Cultures:** The experiments involving the laboratory animals were approved by Ethics Committee of Yerevan State Medical University after M. Heratsi, No 7-26.04.2012: Research is not contrary to the Directive 2001/20/EC of The Legal Aspects of Research Ethics and Science in European Community.

The Ehrlich ascites carcinoma (EAC) cells and mouse blood leucocytes (MBLs) were isolated and maintained as described previously<sup>18, 20, 21</sup>. The mouse pancreatic  $\beta$ -cells were isolated from adult mice pancreas as described elsewhere<sup>22, 23</sup>.

To isolate the hippocampal cells, the rat hippocampus was transferred to 15-ml tubes, adjusted to 2 ml with phosphate-buffered saline, pH 7.4, supplemented with 0.6% glucose and 2 mg/mL of filter-sterilized papain. The tissue was digested at 30°C for 30 min, mechanically dissociated by Pasteur pipette and centrifuged to remove debris. After supernatant centrifugation, the precipitated cells were suspended in the cultivation medium<sup>24</sup>. The concentration of all cells was adjusted to  $5 \times 10^5$  cells/ml and cultivated in the humidified chamber with 5% CO<sub>2</sub> at 37 °C. The EAC cells and MBLs were cultured in suspension. The pancreatic  $\beta$ -cells and hippocampal cells were cultured in poly-D-lysine coated 96-well plates.

In the experiments with plant preparations, their solutions in 20% ethanol were added to the culture medium up to the definite final concentration. The final concentration of ethanol in the cultivation medium was 0.1%. In the special experiment, it was shown that the ethanol up to 1% did not affect the viability of cells. The cell samples, cultivated in 0.1% ethanol containing a medium, served as negative controls (NC). The amounts of viable cells were determined by trypan blue exclusion test<sup>25</sup>.

**Statistical Analysis:** Calculation of IC<sub>50</sub> values was done using GraFit software. The data, obtained at least in three independent experiments, were analyzed using the statistical software InStat,

version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The results are expressed as means  $\pm$  standard errors of means (SEM).

## RESULTS AND DISCUSSION:

**Purification of AD:** Our previous qualitative chemical analysis of ethanol extract of the leaves of *Rumex crispus*<sup>18</sup> showed that the main chemical compounds in it were the flavonoids and the anthracene derivatives, by the literature data<sup>26</sup>. It also contained significant amounts of tannins, coumarins and phloroglucides, and smaller amounts of phenol glycosides and alkaloids. No cardioglycosides and saponins were found. The similar chemical composition was observed for the ethanol extract of the leaves of *Rumex confertus*, prepared as described in the section Materials and Methods.

The two-step low-pressure liquid chromatography fractionation of ethanol extract of SL and separation of AD fraction is described in detail in the section Materials and Methods.

First of all, the SL extract and the isolated from it AD fraction were characterized by TLC analysis using two solvent systems, appropriate for anthracene derivatives (see Materials and Methods). In the TLC pictures, obtained for crude ethanol extract of SL, several AD spots were visible. In the fraction taken after gel-filtration on a column with Sephadex LH-20 most of these spots absent. In the result of second gel-filtration on a column with G-25 Sephadex, only one spot in the mobile phase front was observed, evidencing the purity of the fraction.

The purity of the final AD fraction, eluted from G-25 column with 70% ethanol, was also proved by NMR spectra. <sup>1</sup>H and <sup>13</sup>C NMR spectra of this fraction, presented in **Fig. 1 A** and **B**, respectively, confirm the monomolecular composition of the obtained fraction. In agreement with the reported NMR spectra for emodin from *Caesalpinia decapetala* (Roth) Alston<sup>27</sup>, the characteristics of these spectra allow identifying isolated from SL AD as emodin.

Indeed, the comparison of the absorbance spectra of the purified AD fraction in UV-VIS region (240-600nm) at pH 7.4 and pH 10 **Fig. 2** with the literature data<sup>28, 29</sup> permitted us to reaffirm that it is

emodin. The fluorescence spectra with  $\lambda$  emission = 540 nm and  $\lambda$  excitation = 466 nm were characteristic for emodin too<sup>30</sup>. Hence, on the basis of the obtained characteristics, the anthracene derivative from the ethanol extract of *Rumex confertus* leaves can be recognized as emodin. It is

worth to mention that it was purified with the application of two steps of low-pressure liquid chromatography – a significantly easier procedure as compared with those usually applied for emodin purification<sup>8, 31, 32</sup>. The yield of emodin was 1mg/g of dried extract.

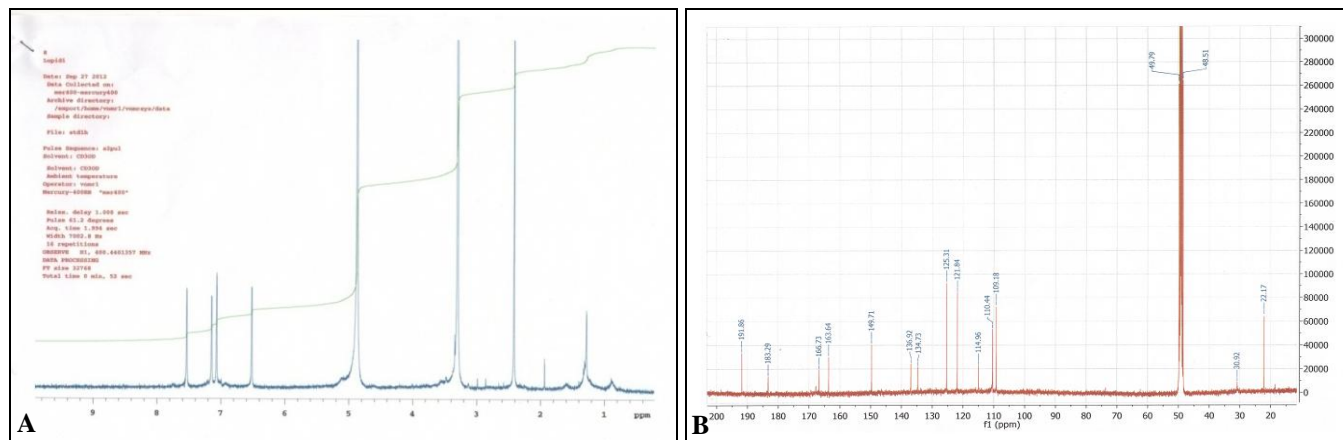


FIG. 1: <sup>1</sup>H (A) AND <sup>13</sup>C (B) NMR SPECTRA OF THE AD FRACTION, ISOLATED FROM *RUMEX CONFERTUS* LEAVES

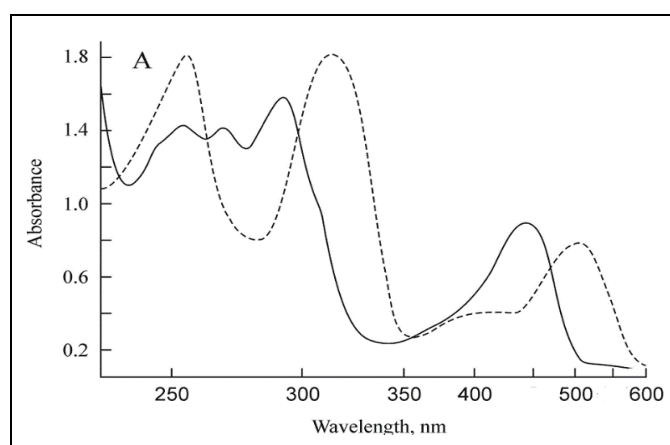


FIG. 2: ABSORBANCE SPECTRA OF AD FRACTION, PURIFIED FROM *RUMEX CONFERTUS* LEAVES AT pH 7.4 (—) AND pH 10 (- - -)

The purity of the final AD fraction, eluted from G-25 column with 70% ethanol, was also proved by NMR spectra. <sup>1</sup>H and <sup>13</sup>C NMR spectra of this fraction, presented in **Fig. 1 A** and **B**, respectively, confirm the monomolecular composition of the obtained fraction. In agreement with the reported NMR spectra for emodin from *Caesalpinia decapetala* (Roth) Alston<sup>27</sup>, the characteristics of these spectra allow identifying isolated from SL AD as emodin.

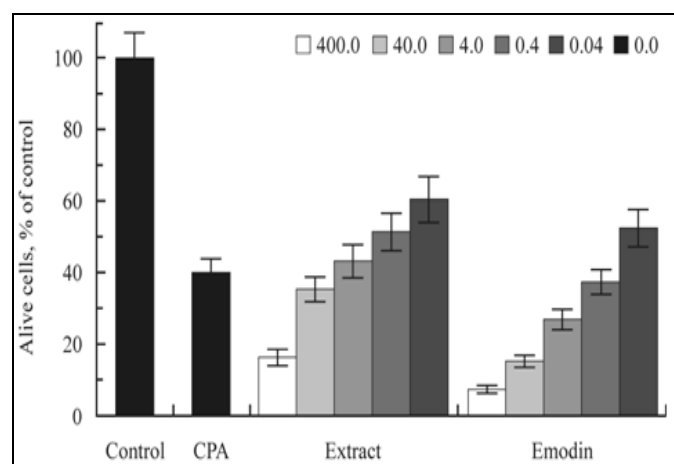
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**Cytotoxicity of Emodin and Ethanol extract from Leaves of *Rumex confertus* Towards Cancer Cells:** The ethanol extracts from the roots,

leaves, and fruits of six *Rumex* species have been screened *in-vitro*, and their remarkable cytotoxic activities on three cancer cell lines *via* apoptosis have been revealed<sup>33</sup>.



**FIG. 3: INFLUENCE OF EMODIN AND ETHANOL EXTRACT OF LEAVES OF *RUMEX CONFERTUS* ON THE VIABILITY OF EAC CELLS**

We studied the influences of ethanol extract and emodin from SL on the growth of EAC cells and non-cancerous MBLs. The concentrations of plant preparations in the experiments were in the interval of 0.04- 400 $\mu$ g/mL. The amounts of living cells after two-day cultivation were expressed as a percentage of NC. As a positive control, the antitumor agent cyclophosphamide is used in concentration 1.3 $\mu$ g/mL. In **Fig. 3**, the results of two-day cultivation of EAC cells in the presence of different concentrations of emodin and the extract are presented as the means  $\pm$  SEM of three independent experiments. The picture demonstrates the high toxicity of plant preparations to these cells. In the same experiments, the MBLs were completely indifferent to the action of both plant preparations.

The high toxicity of SL extract toward cancerous EAC cells compared with the low toxicity toward non-cancerous MBLs was proved by the IC<sub>50</sub> values in inhibiting their growth: 0.3  $\pm$  0.04  $\mu$ g/mL and 25  $\pm$  10 mg/mL, respectively. The IC<sub>50</sub> values for emodin toxicity toward EAC cells and MBLs differed significantly also: 40  $\pm$  10 ng/mL and 0.2  $\pm$  0.1 mg/mL, respectively.

Together with the earlier published antitumor characteristics of different parts of *Rumex* family plants<sup>33</sup>, *Rumex confertus* leaves might be regarded

also as a very hopeful source for anticancer therapeutics.

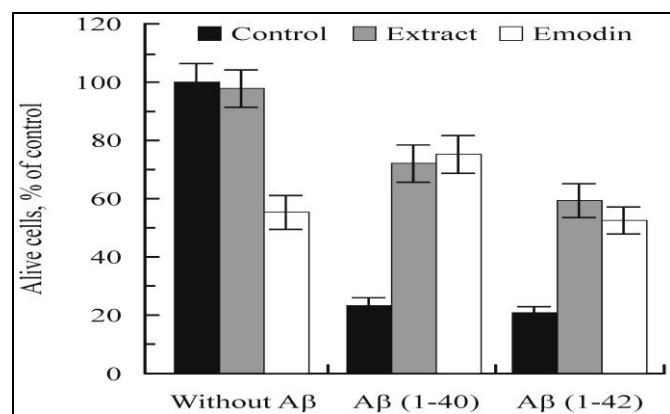
### Protection the Hippocampal cells by Emodin and Ethanol extract from Leaves of *Rumex confertus*:

It is known that the cultivation of primary hippocampal cells in the presence of aggregated amyloid A $\beta$  (1-40/42) peptides resulted in their death<sup>34</sup>. Indeed, in our experiments, the number of living cells in the control samples (59.8 $\times$ 10<sup>5</sup>  $\pm$  4.0) decreased on average to 22.7% (13.6 $\times$ 10<sup>5</sup>  $\pm$  1.9) after 3-day cultivation in the medium, containing 2  $\mu$ M A $\beta$  (1-40), pre-aggregated during 7 days at 37  $^{\circ}$ C in 10 mM HEPES buffer, pH 7.4. In the presence of 0.2  $\mu$ M A $\beta$  (1-42), pre-aggregated during 5 days at the same conditions, the number of cells decreased on average to 20.2% (12.1 $\times$ 10<sup>5</sup>  $\pm$  1.5) ( $p$ <0.0001).

We studied the ability of the SL extract and the emodin from it to protect the hippocampal cells from toxic actions of aggregated amyloid peptides. In **Fig. 4**, the influences of 13.5  $\mu$ g/mL emodin and 30  $\mu$ g/mL SL extract on the viability of hippocampal cells are shown. The number of living cells after three-day cultivation in the absence and presence of aggregated peptides are expressed as the percentage of NC. Here, the means  $\pm$  SEM of five independent experiments are shown.

Interestingly, in the absence of amyloid peptides, emodin at the concentration of 13.5 $\mu$ g/mL *per se* suppressed the number of living cells by more than 40% proving the cytotoxicity of emodin on neuronal cells<sup>12</sup>. However, in the presence of aggregated A $\beta$  (1-40) and A $\beta$  (1-42) peptides, which suppress the growth of hippocampal cells to nearly 20% (positive controls), emodin prevented the death of the cells significantly.

The results for the samples, containing both the plant preparations and the aggregated peptides simultaneously, evidence that both of the plant preparations protected the hippocampal cells from killing by peptides. IC<sub>50</sub> values for this protection in the presence of A $\beta$  (1-40) and A $\beta$  (1-42) by the extract were 14.03  $\pm$  0.13 and 2.6  $\pm$  0.8 $\mu$ g/mL, respectively. The IC<sub>50</sub> value for the protection of hippocampal cells by emodin in the presence of A $\beta$  (1-40) was 2.4  $\pm$  0.02 $\mu$ g/mL.



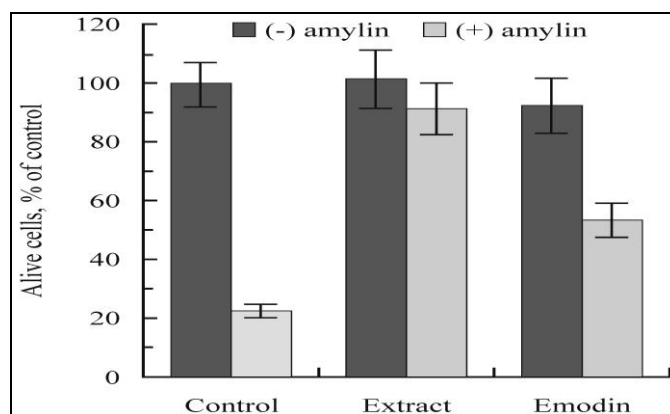
**FIG. 4: INFLUENCE OF EMODIN AND ETHANOL EXTRACT OF LEAVES OF RUMEX CONFERTUS ON THE VIABILITY OF HIPPOCAMPAL CELLS.**

These results demonstrated the *in vitro* protection of hippocampal cells from the toxicity of aggregated A $\beta$  (1-40) and A $\beta$  (1-42) peptides by both the SL extract and the emodin from it. It is in line with the reported protection by emodin from *Polygonum cuspidatum* of cortical neurons from the toxicity of aggregated A $\beta$  (25-35) peptide<sup>12</sup>.

#### Protection the Pancreas Islet $\beta$ -cells from Aggregated Amylin by Emodin and SL Extract:

The effective *in-vitro* inhibition of elevated in diabetes mellitus dipeptidyl peptidase IV and adenosine deaminase by the water extracts from blackberry, melilot, oregano, St. John's wort, seabuckthorn, sorrel, etc. has been observed in our previous work<sup>35</sup> suggesting the expediency of combination of these plants with the antidiabetic drugs in the treatment of type 2 diabetes.

As it is known, the aggregated pancreas peptide hormone amylin is cytotoxic toward pancreas islet  $\beta$ -cells and may be a cause of their loss in type 2 diabetes<sup>36</sup>. In our three-day experiments, the number of  $\beta$ -cells in the control samples ( $40.2 \times 10^5 \pm 2.6$ ) decreased to 20% and lower ( $7.8 \times 10^5 \pm 0.8$ ) in 2  $\mu$ M aggregated amylin containing positive control<sup>23</sup> ( $n = 10$ ,  $p < 0.0001$ ). We studied the ability of the SL extract and the emodin from it to protect  $\beta$ -cells from killing by aggregated amylin. The results obtained in ten independent experiments are shown in **Fig. 5**. In these experiments, the concentration of the extract was 0.7  $\mu$ g/mL and of emodin - 0.3  $\mu$ g/mL. The amounts of living cells in the samples are expressed as the percentage of NC. As a positive control served the sample cultivated three days in the presence of 2  $\mu$ M aggregated amylin.



**FIG. 5: INFLUENCE OF EMODIN AND ETHANOL EXTRACT OF LEAVES OF RUMEX CONFERTUS ON THE VIABILITY OF ISLET  $\beta$ -CELLS**

The data in **Fig. 5** evidence that both the extract and emodin significantly protected  $\beta$ -cells from amylin-induced death. The extract from SL at the used concentration did not possess any cytotoxicity itself and protected the suppressed by amylin viability of  $\beta$ -cells up to ~90%. The used concentration of emodin in the absence of amylin showed some cytotoxicity (inhibited the growth of cells by ~8%). Meanwhile, in the presence of amylin, it possessed significant protection of viability of cells up to over 50%.

Rather low IC<sub>50</sub> values were estimated for the protection of  $\beta$ -cells by ethanol extract ( $0.026 \pm 0.002$   $\mu$ g/mL) and emodin ( $0.6 \pm 0.2$   $\mu$ g/mL). The higher efficacy of the extract compared with the isolated emodin can be explained by the synergy effect due to the presence of substances, enhancing this activity<sup>37</sup>. These results manifested that the extract and emodin from *Rumex confertus* leaves can protect the cultivated pancreas islet  $\beta$ -cells from killing by aggregated amylin.

**CONCLUSION:** for the first time it was shown that the emodin and the extract from *Rumex confertus* leave in *in-vitro* conditions protected:

1. The hippocampal cells from toxic action of aggregated A $\beta$  (1-40/42) peptides, and
2. The  $\beta$ -cells from aggregated pancreas peptide hormone amylin.

The investigation described in this work demonstrated the extremely high toxicity of emodin, and the extract from *Rumex confertus* leaves towards cancer (at least, EAC) cells.

These results provide a rationale for developing anticancer, neuroprotective and antidiabetic remedies from leaves of *Rumex confertus*.

Besides, in this work, a feasible, economical and efficient technique, easier than the previously described methods<sup>8, 30, 31</sup> was used for isolation of a high-value therapeutic agent emodin with a desirable outcome. This is the first report of emodin isolation from *Rumex confertus* leaves.

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**CONFLICT OF INTEREST:** The authors have declared that there is no conflict of interest.

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