

## **In Vitro Screening for Antitumour Activity of *Clinopodium vulgare* L. (Lamiaceae) Extracts**

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**Aqueous extract of *Clinopodium vulgare* L. showed strong antitumour activity when tested *in vitro* on A2058 (human metastatic melanoma), HEp-2 (epidermoid carcinoma, larynx, human) and L5178Y (mouse lymphoma) cell lines—6 h after treatment disintegration of the nuclei and cell lysis started. Applied at a concentration of 80 µg/ml it reduced the cell survival to 1.0, 5.6 and 6.6%, respectively. The concentrations of aqueous extract inhibiting the growth of A2058, HEp-2 and L5178Y cells by 50% (IC<sub>50</sub> values) were calculated to be 20, 10 and 17.8 µg/ml respectively. Two groups of active substances were detected: the first one, probably combining glycosides, influenced adhesion, while the second one caused massive cell vacuolisation. The chloroform extract, which contained ursolic acid and gentriacontan had also cytotoxic, however a little bit weaker effect. All changes observed were irreversible.**

**Key words** antitumour activity; *Clinopodium vulgare* L.; plant extract

For many years plants have been used as therapeutic resources—either as herbal teas or other home made remedies, or as crude extracts or “standard enriched fractions” in pharmaceutical preparations such as tinctures, fluid extracts, powders, pills and capsules.<sup>1)</sup> Extracts from a broad spectrum of plant species contain substances that possess antitumour activity.<sup>2–8)</sup> Most of the active compounds in these extracts still remain unidentified and their presence is detected by biological tests only.<sup>2,3,9,10)</sup> The structure and the mechanism of action of others have been elucidated<sup>11,12)</sup> and some of them are currently used as drugs in chemotherapy (rubomycine, vinblastine, vincristine, colchamine, VM-26 *etc.*). Most of the identified compounds are products of plant secondary metabolism and belong to the classes of alkaloids,<sup>13)</sup> polyphenols,<sup>14)</sup> triterpenes<sup>15)</sup> or are steroid glycosides.<sup>16–19)</sup> The species from genus *Clinopodium* (Lamiaceae) contain a number of triterpenes and triterpenoid saponins<sup>20–27)</sup> as well as some other bioactive substances.<sup>20,22,28)</sup> In Bulgaria *Clinopodium vulgare* L. is a well known medicinal plant mainly used for healing wounds and treating warts due to virus infection. Recent investigations proved its broad-spectrum antibacterial activity.<sup>29)</sup> Several bioactive substances have been identified,<sup>20,30)</sup> of which the saturated hydrocarbon gentriacontan (C<sub>31</sub>H<sub>64</sub>), extracted with chloroform has been proved to have antitumour properties when tested on Ehrlich ascitic and Lewis pulmonary tumour cells, as well as on permanent cell lines of human lymphatic cells MOLT-4 and K-562.<sup>30)</sup> With the experiments reported in this paper we tried to find out whether extracts from *Clinopodium vulgare* L. have selective cytotoxic effect when incubated *in vitro* with normal and cancer cell lines and whether there are another compounds different from gentriacontan that could be related to the antitumour activity of *Clinopodium vulgare* L.

### **MATERIALS AND METHODS**

**Preparation of Extracts** *Clinopodium vulgare* L. (Lamiaceae) was collected on June—July 2000 in Rhodope mountain (Bulgaria). It was authenticated by Dr. Rumen

Mladenov, Department of Botany, University of Plovdiv. A voucher specimen (PHC 2000 L 71) is deposited in the herbarium of the same department. Aqueous extract from dried blades (10% w/v) was prepared by boiling at 100 °C for 5, 15 or 30 min. An aliquot of 5 ml (15 min extraction time) was applied on a column (16 cm×1.3 cm i.d.) of Silica gel 60 (particle size 0.063–0.200 mm, 70–230 mesh ASTM, Merck) and the adsorbed material—eluted successively with 30 ml of each chloroform, chloroform:methanol (9:1 v/v), ethyl acetate and acetone. Fractions of 2.5 ml were collected throughout at a flow rate of 0.2 ml/min. A second aliquot of 5 ml of the same sample was loaded onto a Sephadex G 10 (Pharmacia) column (22 cm×1.6 cm i.d.) The elution was performed with water at a flow rate of 0.15 ml/min and 5 ml fractions were collected. Another 10 g of plant material were subjected to successive extraction with chloroform, chloroform:methanol (9:1 v/v), ethyl acetate and acetone (100 ml of each) in a water bath at 60, 65, 77 and 56 °C respectively, for 2 h. The extracts were left at room temperature, filtered through paper filter and then concentrated under vacuum. The samples were sterilised through a Millipore 0.22 µm filter and stored at 4 °C.

Thin layer chromatography (TLC) was performed on Silica gel 60 F<sub>254</sub> aluminium sheets (Merck) using one of the following solvent systems: diethyl ether:toluene (2:1 v/v), chloroform:methanol (9:1 v/v); chloroform:methanol:acetic acid (2:7:1 v/v). Spots were detected by iodine vapour, carbonisation (H<sub>2</sub>SO<sub>4</sub>:CH<sub>3</sub>OH 1:1 v/v, heating for 10 min at 100 °C) or anisaldehyde reagent.<sup>31)</sup> All chemicals used were of analytical grade.

**Cell Culture Methods** Cell lines HEp-2 (epidermoid carcinoma, larynx, human, ATCC CCL 23), A2058 (human metastatic melanoma, ECACC 91100402), L5178Y (mouse lymphoma cells, wild type, NBIMCC 101), FL (normal amniotic cells, human, ATCC CCL 62) and 3T3 (embryonic fibroblasts, mouse, ATCC CCL 92) were used in the experiments. Cells were cultivated in liquid DMEM:Ham's F12 (1:1) medium (Serva), supplemented with 10% (v/v) calf serum, 100 IU penicillin and 100 µg/ml streptomycin in Her-

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aeus incubator at 37 °C with 5% CO<sub>2</sub> in air and high humidity. L5178Y cells were cultivated in RPMI-1640 medium supplemented with amino acids under the conditions mentioned above. Trypsin treatment and subculturing were done according to the adapted Invitox protocols.<sup>32,33</sup> Cell density was determined by a standard haemocytometer chamber. Cell viability was measured with the trypan blue exclusion test.<sup>34</sup>

**Neutral Red (NR) Test** The NR assay based on the incorporation of dye into the lysosomes of viable cells after incubation with test agents, was carried out as previously described.<sup>35</sup> Briefly, the cells were then washed with a formalcalcium solution (1% anhydrous CaCl<sub>2</sub> w/v in 0.4% formaldehyde) which removed the dead cells. The dye was then extracted from the intact cells with an acetic acid–ethanol solution (1% glacial acetic acid in 50% ethanol). The absorbance of the solution was read at 540 nm.

Percentage of cytotoxicity (PC) was calculated as follows:

$$PC (\%) = [1 - (\text{Abs}_{540\text{nm}}^{\text{test}}) / (\text{Abs}_{540\text{nm}}^{\text{control}})] \times 100$$

**In Vitro Effects of *C. vulgare* L. Extracts on Cancer and Normal Cell Lines** HEP-2, A2058, L5178Y, FL and 3T3 cells (1 × 10<sup>5</sup> cells/ml) were plated in 96-well plates or cell culture flasks with a growth area of 25 cm<sup>2</sup>. Twenty four hours later they were treated with aqueous extract at a final concentration of 20, 40, 60 or 80 µg/ml. Cell viability, counted in haemocytometer chamber, was checked every 24 h up to 96 h. Cells were also inspected on an inverted microscope in 24 h intervals until monolayers were formed for changes in their shape, level of adhesion and some other morphological alterations caused by the treating agent. In addition the experiment was repeated in 5 cm petri dishes with glass lamellae. Every 2 h up to 12 h an aliquot of the treated with aqueous extract cells were fixed in methanol for 7 min and stained with hematoxylin-eosin. The reversibility of the effects was evaluated in the following manner: 4 h after treatment the extract was eliminated, cells were trypsinised and seeded in a fresh tissue culture medium. Doubling time was calculated as described.<sup>36</sup>

Results were statistically processed and presented as mean values of 3 independent experiments with 3 replicates per experiment.

## RESULTS

In Bulgarian traditional medicine an aqueous *Clinopodium vulgare* L. extract prepared by boiling grounded blades for 5 min is recommended for antitumour treatment. In our experiments we applied the same recipe for preparation. Visual evaluation of the effect of treatment with this extract on HEP-2, A2058, L5178Y, FL and 3T3 cells showed that the toxic effect is selective and can be observed very early. While the normal FL and 3T3 cells were not different in any way from the controls, cells from the cancer cell lines underwent massive alterations. Four hours after treatment A2058 and HEP-2 cells began to round up with vacuolisation of the cytoplasm (Fig. 1). Two hours later the level of adhesion was influenced—many cells detached easily from the plastic flasks and moved into medium. At the same time disintegration of the nuclei and cell lysis started; these could be observed for a period of 2 h—between 6 and 8 h after treatment. Dividing cells were completely absent. Cells from the mouse lymphoma

line L5178Y were also affected very quickly, forming grape-shaped clusters (Fig. 2) and 24 h after treatment with 80 µg/ml aqueous extract only 8.47% of them survived.

All changes and cell damages were irreversible—elimination of the extract 4 h after treatment, followed by trypsinisation and transfer the cells in a fresh culture medium did not restore their normal status. They were still unable to attach to the plastic flasks and formed multicellular aggregates, which floated in the medium. Two hours later they died.

When aqueous extract was applied at the beginning of incubation, alterations were even more drastic—the cells did not attach to the flask area at all and quickly died.

Results showed that the cell viability is concentration- and time-dependent. For L5178Y cells this dependency was valid for all concentrations and the whole period tested (Table 1), while for HEP-2 and A2058 cell lines the effect of 40, 60 and 80 µg/ml extracts equalised at 72 h (Tables 2, 3). Ninety six hours after treatment with 80 µg/ml aqueous extract, the survival of L5178Y, HEP-2 and A2058 cells was 6.6, 5.6 and 1.0%, respectively. At the same time *C. vulgare* cytotoxic effect on FL and 3T3 cells was insignificant, if any (Tables 4, 5). Their doubling time was calculated for 48—72 h interval, which corresponded to the logarithmic cell growth. For cell line FL it was 13.5, 13.6, 14.0 and 13.0 h for the fourth variants of extract concentrations applied and 13.3 h for untreated cells. For the 3T3 cells these values were 14.5, 14.4, 13.7, 14.8 and 14.3 h respectively. The NR assay confirmed the selectivity of the cytotoxic effect of the aqueous extract from *Clinopodium vulgare*. Treatment with an extract concentration of 80 µg/ml for 24 h showed significant cytotoxicity on L5178Y, A2058 and HEP-2 cells (89, 93 and 84% respectively) while the viability of the normal cells (FL and 3T3) was not significantly affected compared to the control—16% and 11% respectively (Fig. 3).

IC<sub>50</sub> values for L5178Y, HEP-2 and A2058 cells were calculated to be 17.8 µg/ml (for L5178Y cells), 10 µg/ml (for HEP-2 cells) and 20 µg/ml (for A2058 cells), respectively.

The antitumour agents of *C. vulgare* were almost completely extracted by boiling grounded blades in water for 5 min. Extended extraction for up to 30 min did not show significant decrease of cell survival percentage. Figure 4 illustrates this effect on the example of A2058 cells. The bioactive substances were eluted from the Sephadex G10 column in fraction 7, which combines molecules with molecular mass between 370 and 485 Da.

When an aliquot of aqueous extract was passed through a Silica gel column, and the adsorbed material eluted successively with a series of organic solvents (see Materials and Methods), the antitumour active substances were recovered in fractions 13—17 (elution with chloroform/methanol, 9/1) and 26—27 (elution with ethyl acetate). Besides, components eluted with a less polar solvent system caused massive detachment of cells from the plastic flasks; twenty-four hours after treatment none of the cells was alive and the dead cells formed conglomerates. The effect produced by the substances desorbed with ethyl acetate was different—all cells were attached to the flask surface, but strong vacuolisation of their cytoplasm was observed. Up to 48 h (e.g. 24 h later) most cells were dead, nevertheless they still retained their adhesive properties.

Successive extraction of the herbal material with chloro-

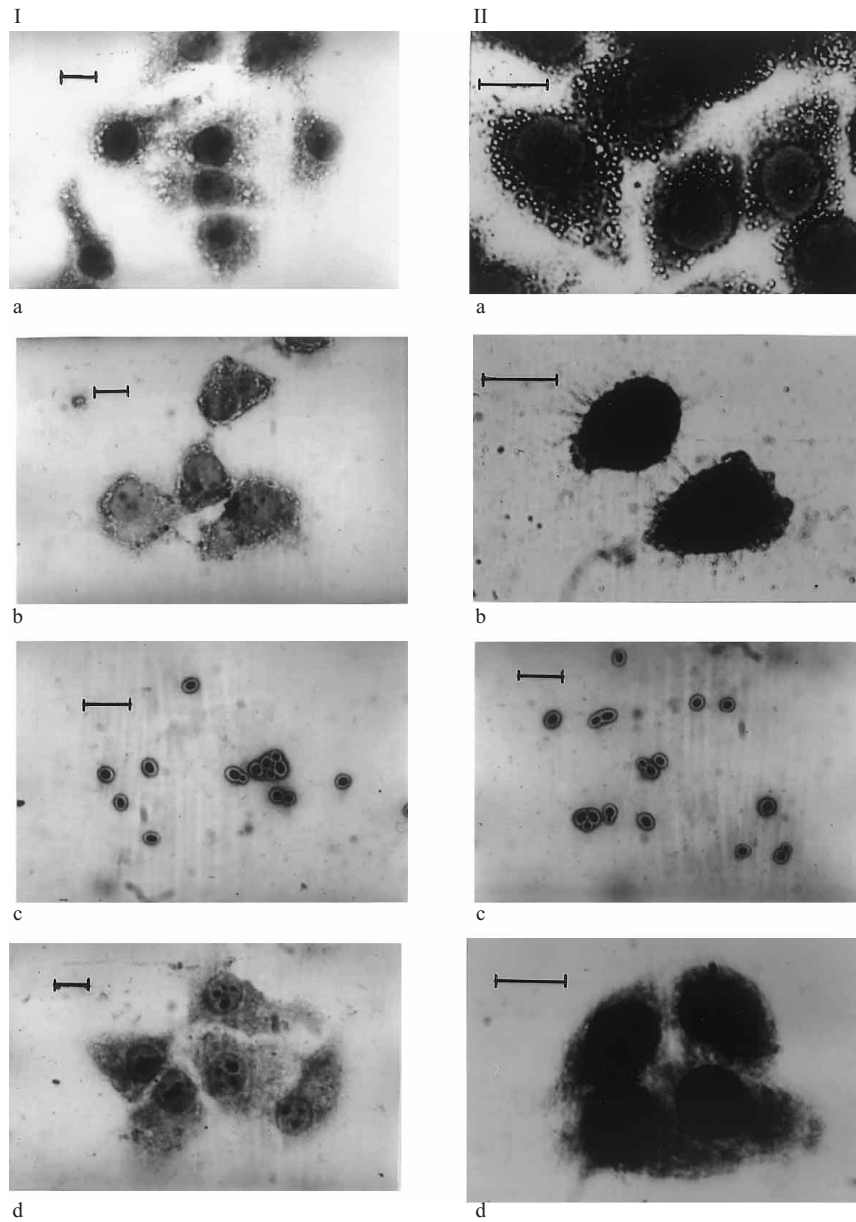


Fig. 1. Effect of Aqueous Extract (20  $\mu\text{g/ml}$ ) on HEP-2 Cells (I) and A2058 Cells (II) 4 h after Treatment  
 (a) Vacuolisation of the cytoplasm, (b) round-shaped cells, (c) detached cells, (d) control. Bar represents a scale of 10  $\mu\text{m}$ .

form, chloroform/methanol (9/1), ethyl acetate and acetone under heating in water bath at appropriate temperature gave 4 fractions. The first two of them only possessed antitumour activity, with second being more active than the chloroform one. Associated with the treatment changes in cell morphology were identical to those, observed for the aqueous extract.

The composition of chloroform and chloroform/methanol fractions was compared with those of the combined 13—17 and 26—27 fractions, designated as fraction CM and fraction EA, respectively. On TLC aluminium sheets developed with a solvent system diethyl ether:toluene (2:1 v/v) fraction CM gave a main spot with  $R_f$  0.67. The positive detection by anisaldehyde reagent suggests its glycoside nature. Two spots with  $R_f$  0.83 and 0.93 were detected in fraction EA when solvent system chloroform: methanol (9:1 v/v) was used, and one main with yellow colour and  $R_f$  0.89 when the sheet was developed with chloroform: methanol: acetic acid (2:7:1

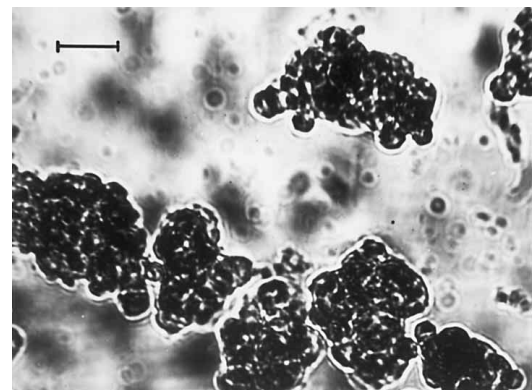


Fig. 2. Effect of Aqueous Extract (20  $\mu\text{g/ml}$ ) on L5178Y Cells 24 h after Treatment  
 Bar represents a scale of 10  $\mu\text{m}$ .

Table 1. Cytotoxic Effect of Aqueous Extract on L5178Y (Mouse Lymphoma Cells)

Concentration of the extract ( $\mu\text{g/ml}$ )	L5178Y treatment							
	24 h		48 h		72 h		96 h	
	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)
0	118 $\pm$ 0.2	100	146 $\pm$ 0.8	100	120 $\pm$ 0.3	100	122 $\pm$ 1.1	100
20	96 $\pm$ 0.8	81.36	80 $\pm$ 1.1	54.79	66 $\pm$ 0.3	55.00	56 $\pm$ 0.4	45.90
40	30 $\pm$ 0.2	25.42	16 $\pm$ 0.7	10.96	16 $\pm$ 0.3	13.33	14 $\pm$ 0.9	11.48
60	26 $\pm$ 0.4	22.03	14 $\pm$ 0.2	9.59	14 $\pm$ 0.4	11.67	12 $\pm$ 0.8	9.84
80	10 $\pm$ 0.6	8.47	9 $\pm$ 0.3	6.16	8 $\pm$ 1.2	6.67	8 $\pm$ 0.6	6.56

Initial density  $1 \cdot 10^5$  cells/ml. Exposure time 24, 48, 72 and 96 h. The results are means $\pm$ S.D., ( $n=9$ ).

Table 2. Cytotoxic Effect of Aqueous Extract on HEP-2 (Epidermoid Carcinoma, Larynx, Human) Cells

Concentration of the extract ( $\mu\text{g/ml}$ )	HEP-2 treatment							
	24 h		48 h		72 h		96 h	
	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)
0	32 $\pm$ 0.6	100	44 $\pm$ 0.9	100	68 $\pm$ 0.9	100	72 $\pm$ 0.4	100
20	20 $\pm$ 1.2	62.50	16 $\pm$ 0.2	36.36	8 $\pm$ 0.1	11.76	4 $\pm$ 0.2	5.56
40	12 $\pm$ 0.8	37.50	9 $\pm$ 0.7	20.45	5 $\pm$ 0.4	7.35	3 $\pm$ 0.7	4.17
60	8 $\pm$ 1.1	25.00	7 $\pm$ 1.1	15.91	5 $\pm$ 1.3	7.35	4 $\pm$ 1.6	5.56
80	7 $\pm$ 0.9	21.88	6 $\pm$ 0.8	13.64	6 $\pm$ 0.9	8.82	4 $\pm$ 1.2	5.56

Initial density  $1 \cdot 10^5$  cells/ml. Exposure time 24, 48, 72 and 96 h. The results are means $\pm$ S.D., ( $n=9$ ).

Table 3. Cytotoxic Effect of Aqueous Extract on A 2058 (Amelanotic Melanoma, Human) Cells

Concentration of the extract ( $\mu\text{g/ml}$ )	A 2058 cells treatment							
	24 h		48 h		72 h		96 h	
	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)
0	64 $\pm$ 0.3	100	110 $\pm$ 1.2	100	174 $\pm$ 0.1	100	198 $\pm$ 1.1	100
20	32 $\pm$ 1.2	50.00	18 $\pm$ 0.8	16.36	9 $\pm$ 1.1	5.17	6 $\pm$ 0.4	3.03
40	20 $\pm$ 1.2	31.25	12 $\pm$ 0.6	10.91	5 $\pm$ 0.8	2.87	3 $\pm$ 1.2	1.52
60	8 $\pm$ 1.3	12.50	8 $\pm$ 0.5	7.27	6 $\pm$ 0.9	3.45	4 $\pm$ 0.8	2.02
80	6 $\pm$ 1.1	9.38	6 $\pm$ 0.8	5.45	4 $\pm$ 0.5	2.30	2 $\pm$ 0.6	1.01

Initial density  $1 \cdot 10^5$  cells/ml. Exposure time 24, 48, 72 and 96 h. The results are means $\pm$ S.D., ( $n=9$ ).

Table 4. Effect of Aqueous Extract on FL (Normal Human Amnion) Cells

Concentration of the extract ( $\mu\text{g/ml}$ )	FL cells treatment							
	24 h		48 h		72 h		96 h	
	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)
0	57 $\pm$ 1.6	100	87 $\pm$ 1.6	100	127 $\pm$ 1.6	100	166 $\pm$ 0.3	100
20	55 $\pm$ 1.5	96.49	80 $\pm$ 2.1	91.95	119 $\pm$ 2.1	93.70	158 $\pm$ 2.4	95.18
40	52 $\pm$ 0.5	91.23	79 $\pm$ 0.4	90.80	125 $\pm$ 0.3	98.43	161 $\pm$ 0.6	96.99
60	55 $\pm$ 2.1	96.49	71 $\pm$ 1.2	81.61	117 $\pm$ 1.3	92.13	148 $\pm$ 1.4	89.16
80	45 $\pm$ 1.7	78.95	73 $\pm$ 0.7	83.91	115 $\pm$ 0.8	90.55	132 $\pm$ 1.7	79.52

Initial density  $1 \cdot 10^5$  cells/ml. Exposure time 24, 48, 72 and 96 h. The results are means $\pm$ S.D., ( $n=9$ ).

Table 5. Effect of Aqueous Extract on 3T3 (Embryonic Mouse Fibroblasts) Cells

Concentration of the extract ( $\mu\text{g/ml}$ )	3T3 cells treatment							
	24 h		48 h		72 h		96 h	
	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)	Live cells/ $\text{ml} \cdot 10^4$	Survival (%)
0	$38 \pm 0.7$	100	$56 \pm 0.6$	100	$88 \pm 1.2$	100	$96 \pm 1.8$	100
20	$38 \pm 0.4$	100	$48 \pm 1.4$	85.71	$79 \pm 2.2$	89.77	$88 \pm 0.4$	91.67
40	$36 \pm 1.2$	94.74	$52 \pm 1.1$	92.86	$82 \pm 0.8$	93.18	$94 \pm 0.6$	97.92
60	$32 \pm 2.2$	84.21	$44 \pm 1.6$	78.57	$82 \pm 0.6$	93.18	$85 \pm 1.2$	88.54
80	$34 \pm 0.3$	89.47	$45 \pm 1.3$	80.36	$74 \pm 2.4$	84.09	$82 \pm 1.5$	85.42

Initial density  $1 \cdot 10^5$  cells/ml. Exposure time 24, 48, 72 and 96 h. The results are means  $\pm$  S.D., ( $n=9$ ).

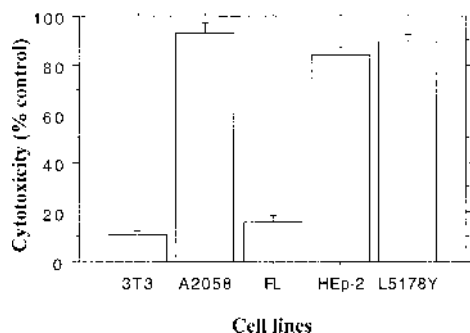


Fig. 3. Cytotoxic Effect of Aqueous Extract on L5178Y, HEp-2, A2058, FL and 3T3 Cells Assessed by the Neutral Red Assay

Cells were treated with  $80 \mu\text{g/ml}$  aqueous extract for 24 h. Results are given as means of 6 replicates.

v/v). All these compounds were found in chloroform/methanol extract. Fractions CM and EA did not contain ursolic acid or genticic acid, as judged by the comparison with the relevant references. These substances were isolated during the first extraction step—with chloroform.

## DISCUSSION

Results from light microscopic observation, NR and proliferation tests showed unambiguous cytotoxic effects and antitumour activity of aqueous *Clinopodium vulgare* L. extract on treated *in vitro* cells. The extract caused typical changes in the shape of the examined cancer cells, altered their ability to attach to plastic flasks and to form monolayers. Considerable reduction in the number of the viable cancer cells was observed together with irreversible structural damages—vacuolisation of the cytoplasm, disintegration of the nuclei and lysis of the cell. These changes caused cell death, which set in as early as the first hours of treatment.

The effect of aqueous extract shows strong selectivity—percentage of survival of normal FL and 3T3 cells is very high. This suggests that the active substances interact with specific tumour-associated receptors, thus triggering some mechanisms that cause cell death. Taking into account the correlation between cell survival and concentration of the extract/time of treatment, it could be assumed that either the receptors are identical and differences in the final outcome among the cell lines tested reflect differences in their origin and physiology or the receptors are similar, and those specific for A2058 cells show highest affinity to the bioactive

substances in aqueous extract.

In aqueous extract of *C. vulgare* L. there are at least two groups of natural products with antitumour properties. The first one, eluted from Silica gel column with chloroform:methanol (9:1 v/v) probably consists of one (or more?) substances, belonging to the class of glycosides, as judged by the positive detection with anisaldehyde reagent. The second group combines more polar compounds—they were desorbed from Silica gel resin with ethyl acetate ( $D_{EA} (6.02) > D_{CM} (5.4)$ ). These two groups of substances differ not only in their chemical properties (at least solubility), but in their biological effect as well. The glycoside group affect some adhesion factors as it causes massive detachment of the cells from the plastic flasks. Several saikosaponin homologues, called clinoposaponins have been isolated from *C. vulgare* and their structure elucidated on the basis of spectral and chemical evidences.<sup>21</sup> One could argue that the molecular mass of saikosaponins is higher than 485 Da, while the antitumour compounds according to our results from gel-filtration chromatography have to have molecular mass between 370 Da and 485 Da. However it has to be beared in mind that the tightly cross-linked structure of Sephadex G10 does not exclude ionic and aromatic interactions with certain molecular species, thus causing their later elution, and hence lower predicted molecular mass.

As regards to the second group of *C. vulgare* bioactive substances in the aqueous extract—those eluted with ethyl acetate—the mechanism of their effect is different, with vacuolisation being the main feature of this effect. Some studies to elucidate the structure of these compounds are now in progress.

Two other substances with proved antineoplastic properties are present in chloroform, but not in aqueous extract. Genticic acid—a linear saturated hydrocarbon ( $C_{31}H_{64}$ ), was reported to inhibit the cell growth of Erlich ascites tumour in mice, and also possesses antitumour activity *in vitro* against CEM and K-562 human leukaemia lines.<sup>30</sup> At present the mechanism of its action is unknown. Much more is known about the second compound—ursolic acid. It is a pentacyclic triterpene acid, isolated from a number of plant species<sup>37–39</sup> which induces cell death through apoptosis<sup>40</sup> probably *via* cytochrome C-dependent caspase-3 activation.<sup>41</sup>

In conclusion, several compounds could be related to the antitumour properties of *Clinopodium vulgare* L. Their identification would be of importance for development of new antitumour drugs.

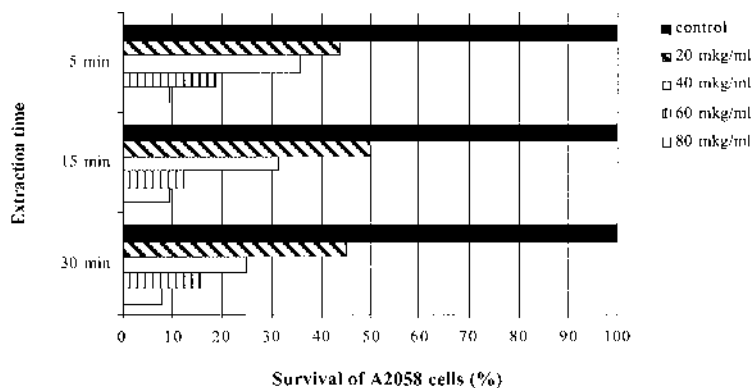


Fig. 4. Relation between the Extraction Time (5, 15, 30 min) and Effect of Aqueous Extract on A2058 Cells 24 h after Treatment

The cell viability is expressed as mean value in percentage of control (based on triplicates).

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