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CYTOTOXIC PROPERTIES OF *CLINOPODIUM VULGARE* L. EXTRACTS ON SELECTED HUMAN CELL LINES

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Abstract

Plants of Lamiaceae family are highly recommended as a source for new pharmaceuticals due to their wide range of biological activities. We have further investigated the in vitro effects of acidified, alkalized and lipophilic extracts of the medicinal plant *Clinopodium vulgare* L. on the cell viability of selected cell lines. In vitro cytotoxicity was evaluated against CaOV (human testis cystadenocarcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colorectal adenocarcinoma) and FL (human amnion normal) cell lines using MTT assay. We found that two of the extracts (acidified and lipophilic) exerted selective dose-dependent cytotoxic activity against CaOV (IC_{50}: 225–260.86 µg/mL) and HeLa cells (IC_{50}: 360.27–388.5 µg/mL), while the cytotoxicity of the alkalized extract against these cell lines was less pronounced. All tested extracts showed very weak or lack of cytotoxic actions towards HT-29 and the normal FL cells. Moreover, these results indicate that *Clinopodium vulgare* extracts possess selective anticancer activity and could serve as a source for isolation and development of new therapeutic anticancer agents.

Key words: *Clinopodium vulgare*, cytotoxicity, anticancer activity, in vitro

Introduction. Medicinal plants belonging to Lamiaceae have been extensively used as sources of new natural products with different modes of action including anticancer activity. The aerial part of these species contains high amounts of essential oils, terpenoids and phenolic compounds [1–4]. Many of the widely used cytostatic drugs (taxol, vincristine, vinblastine, camptothecin) are plant-derived. The usage of natural products with anticancer properties has received considerable attention during the last years, which is due to their low toxicity compared with the conventional chemotherapeutics and lack of undesirable side effects.

Clinopodium vulgare L. (wild basil) is an aromatic herb, which belongs to the family Lamiaceae. Traditionally, it is used for preparation of food dishes and tea, as well as in the Bulgarian folk medicine for treatment of wounds, warts,

prostatitis, mastitis and gastric ulcers. It has been reported that extracts from this species possess antibacterial [5, 6] , anti-inflammatory [7], antioxidant [8, 9] and anticancer activities [10, 11].

In terms of chemical composition, the major components of *Clinopodium vulgare* L. have been identified, including phenolcarboxylic acids, flavonoids, monoterpenoids, diterpenoid quinines, triterpenoid glycosides, and high content of triterpenoid saponins [12–14]. Also, there are some data about the chemical composition of the essential oil of *C. vulgare* L. [15, 16].

In this study, we investigated the cytotoxic activities of three different extracts (acidified, alkalized and lipophilic) isolated from *Clinopodium vulgare* L. towards selected human cancer and normal cell lines.

Materials and methods. ***Plant material and preparation of extracts.*** Aerial parts of the herb *Clinopodium vulgare* L. were collected in Lozenska Mountain (Sofia Region, Bulgaria), near "Saint Spas" Monastery, open grass area with shrubs, 850 m a.s.l., Bulgaria. Date: 01/07/2014, Leg.: Krum Bardarov, Det. Anely Nedelcheva. A voucher specimen is deposited in Herbarium of Sofia University "St. Kliment Ohridski" (SO 107606). The plants were air-dried in clean, dark and airy room and stored in ventilated paper boxes before preparation of the materials for investigation. Plant organs (leaves, flowers and stems) were gently separated and processed individually. Randomly collected parts from these plant organs and from the whole plants were milled/homogenized in a grinder-mill before water extraction, lyophilization and preparation of butanol extracts.

Sixty grams of powdered plant material (leaves, stems, flowers and total plant material) were incubated in a glass beaker with 1000 mL 95 °C HPLC-grade water and gently agitated until cooled at room temperature (approximately 2 hours). Two extraction setups were used. One –with water alkalized with ammonia to pH 9, and the other with water acidified with formic acid to pH 2. The third variant was extraction with pure water at natural pH (5–6). The aqueous extracts were centrifuged, filtered, frozen at $t = -50\,^{\circ}\mathrm{C}$ and then lyophilized.

Further, the plant aerial parts were extracted with supercritical fluid (CO_2) extraction to obtain a DMSO soluble lipid fraction.

For the cytotoxicity tests, acidified and alkalized *Clinopodium* lyophilisates were dissolved in D-PBS (Gibco, Invitrogen, USA), and the lipophilic fraction was dissolved in distilled water containing 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany).

Cytotoxicity screening. In vitro cytotoxicity tests were assessed by using four human cell lines: CaOV (human testis cystadenocarcinoma cells, NBIMCC 1108), HeLa (human cervical adenocarcinoma cells, NBIMCC 164; ATCC CCL 2), HT-29 (human colorectal adenocarcinoma cells, ECACC 91072201; ATCC HTB-38) and FL (human amnion normal cells, NBIMCC 94; ATCC CCL 62). HT-29 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and the other three cell lines from the National Bank for Industrial

Microorganisms and Cell Cultures (NBIMCC). Prior to treatment, the cells were expanded in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Austria) and antibiotics (100 U/mL penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Germany)). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until they reach confluence. Then, they were trypsinized and the concentration of viable cells was determined using the Trypan blue assay. All cells were seeded in 96-well microtiter plates at density 5×10^4 cells/well and incubated overnight to obtain a 70% confluent layer. The monolayer was treated with different concentrations (400, 200, 100, 50, 25, 10 µg/mL) of the examined three *Clinopodium* extracts and incubated for 48 h at 37 °C. To the control wells was added equivalent volume of the solvents: D-PBS (Gibco, Invitrogen, USA) for the acidified and alkalized extracts and dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) at final concentration of 0.1% for the lipophilic extract. All samples were tested in triplicates and repeated at least two times.

Cell viability was determined by MTT assay [17]. During the last 3 h of incubation, 20 µL 5 mg/ml MTT (Sigma-Aldrich, Germany) were added into each well. The supernatants were discarded and the cells were washed with D-PBS. The accumulated formazan was dissolved in 100 µL of DMSO. Optical density was measured at 570 nm on a microplate reader (ELx800™, BioTek). The mean absorbance measured in the control wells was referred as 100% cell viability. Cell viability was calculated as a percentage of corresponding control values (non-treated cells) obtained from two independent experiments performed in triplicates. IC_{50} values were calculated from concentration-response curves.

Statistical analysis. Results are expressed as mean values ± standard deviation (SD). Statistical differences were analyzed by the Mann–Whitney *U*-test using the StatView programme (SAS Institute, Inc.). Values of $P < 0.05$ were considered significant. All results were compared to those from the controls.

Results and discussion. To evaluate the anticancer properties of the *Clinopodium vulgare* extracts, we have selected four continuous human cell lines: CaOV (NBIMCC 1108) established from testis cystadenocarcinoma, HeLa (NBIMCC 164) derived from cervical adenocarcinoma, HT-29 (ECACC 91072201) isolated from colorectal adenocarcinoma, and FL (NBIMCC 94) established from normal amniotic cells. The three cancer cell lines were chosen since in the Bulgarian folk medicine wild basil is recommended for treatment of disorders related to the male and female reproductive systems as well as to the digestive system. To establish potentially selective anticancer activity, we have used the cell line FL as a control, because it is isolated from normal (non-tumour) cells.

Two of the investigated extracts (acidified and lipophilic) showed selective dose-dependent cytotoxic effects on CaOV and HeLa cells (Fig. 1A, B). CaOV cells were much more sensitive to the toxic actions (with IC_{50} values of 260.86

µg/mL for the acidified extract and 225 µg/mL for the lipophilic extract), than HeLa cells (IC_{50}: 360.27-388.5 µg/mL). Both extracts caused significant reduction (44–57%) of cell viability at the highest used concentration (400 µg/mL) (Fig. 1A, B). The alkalized extract induced decreased vitality rate of approximately 28% for CaOV cells and 20% for HeLa cells (Fig. 1A, B). Even at the highest concentration (400 µg/mL), all investigated extracts caused a slight viability reduction of HT-29 and FL cells in a range of 6–18% (Fig. 1C, D).

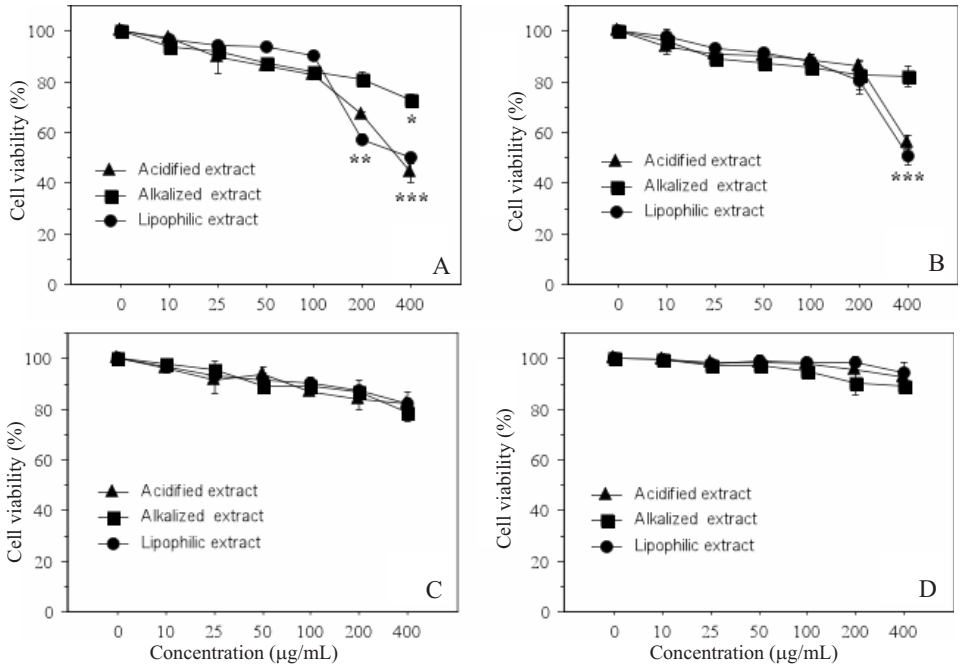

Fig. 1. Cytotoxic effects of *Clinopodium vulgare* extracts. Viability of CaOV (**A**), HeLa (**B**), HT-29 (**C**) and FL (**D**) cells grown for 48 h in the presence of increasing concentrations of the extracts, determined by MTT test. Error bars indicate mean ± SD, $n = 6$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.5$, significantly different compared to the control

The preliminary chemical screening of the acidified *Clinopodium vulgare* extract showed that decarboxilated caffeic acid and its ester, the rosmarinic acid, are the major components [14]. These phenolic compounds are typical for the representatives of Lamiaceae and it has been demonstrated that caffeic acid-containing glycolsides as well as the rosmarinic acid have anticancer activity against different cancer cells influencing several signal transduction pathways [18, 19]. Ursolic acid, gentriacontan and clinoposaponins are other compounds found in *Clinopodium vulgare* extracts [10, 13, 14] that possess anticancer activity [10, 20]. Probably, the obtained viability reduction of CaOV and HeLa cells (Fig. 1A, B) treated with the lipophilic extract is due to these compounds.

Comparing the data from this study with the previous results [10], we may conclude that several compounds are responsible for the observed anticancer prop-

erties of the *Clinopodium vulgare* extract involving different cell signalling pathways. In this study, we have measured the mitochondrial activity by using the MTT-assay, while in the previous study [10] the Neutral red (NR) assay was used , evaluating the lysosomal integrity and activity. This is the reason to get different IC_{50} values: 225–388 µg/mL for the MTT-test and 10–20 µg/mL for the NR-test. In both studies, the investigated extracts exerted selective dose-dependent cytotoxic actions on certain cancer cells and did not affect the normal cells. This reveals that *Clinopodium vulgare* L. is a promising source for isolation of anticancer compounds with selective action. Our experience with isolation and identification of different substances from plant extracts and their assessment for several types of biological activity in vitro showed that the activity is mostly due to a complex mixture rather than to a single compound.

Although, many well-known compounds exhibiting antioxidant and anticancer activity have been isolated, the full chemical composition of the extracts from *Clinopodium vulgare* is more complicated. This is due to the presence of unknown derivatives and conjugates, as well as the lack of reference standards for them. Thus, our study is a solid base for further studies towards isolation of novel substances with anticancer properties and drug development.

Conclusion. Our results showed that *Clinopodium vulgare* extracts possess selective anticancer activity in a concentration dependent manner. The cytotoxic action is mediated by both the mitochondrial and lysosomal induced cell death pathway.

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