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In vitro cytotoxicity of allelopathic plants *Adonis vernalis* L. *Origanum vulgare* ssp. *vulgare* L. and *Nepeta nuda* subsp. *nuda***Vanya Koleva¹, Asya Dragoeva¹, Zheni Stoyanova¹, Zhenia Yordanova², Selime Ali¹, Nikolay M. Uzunov^{1,3}, Laura Melendez-Alafort⁴, Antonio Rosato^{4,5}, Dobromir D. Enchev¹**¹*Faculty of Natural Sciences, Konstantin Preslavski University of Shumen, 115 Universitetska Str., 9712 Shumen, Bulgaria*²*Sofia University, Faculty of Biology, 8 "Dragan Tsankov" Blvd., 1421 Sofia, Bulgaria*³*INFN, Laboratori Nazionali di Legnaro, Legnaro (Padova), Italy*⁴*Veneto Institute of Oncology IOV-IRCCS, Via Gattamelata 64, 35128 Padua, Italy*⁵*Department of surgery, oncology and gastroenterology, University of Padua, Via Gattamelata 64, 35128 Padua, Italy*vanyakolleva@gmail.com

Abstract: Medicinal plants produce various secondary metabolites as a part of their chemical defence and survival in nature. These compounds have a wide range of biological activities. Nowadays, medicinal plants are used as source of allelochemicals and new effective anticancer agents. Our previous studies revealed allelopathic potential of water extracts of *Adonis vernalis* L. (Ranunculaceae), *Origanum vulgare* ssp. *vulgare* L. and *Nepeta nuda* subsp. *nuda* (Lamiaceae). Present study aimed to evaluate the effect of the same extracts in vitro on human hepatoma cell line SK-HEP-1. Cell proliferation/viability was assessed using Premixed WST-1 Cell Proliferation Reagent.

Adonis water extract (1.83mg/ml) had notable negative influence on cancer cell line tested. *Oregano* (3.5 mg/ml) also exerted negative effect, but to a lesser degree. On the contrary, *nepeta* water extract (6.59 mg/ml) had an opposite effect, stimulating cell proliferation. One possible explanation could be the type of extraction: after treatment with *nepeta* methanol extract (6.59 mg/ml) cell viability was significantly reduced.

In conclusion, *Adonis vernalis* and *Nepeta nuda* subsp. *nuda* possess metabolites with growth inhibitory effect on human hepatoma cell line SK-HEP-1. Further research is needed to clarify biological activity of lower concentrations which are appropriate to enable the design of new anticancer drugs.

Keywords: water extracts, human hepatomacell line SK-HEP-1, *Adonis vernalis* L. (Ranunculaceae), *Origanum vulgare* ssp. *vulgare* L. and *Nepeta nuda* subsp. *nuda* (Lamiaceae)

Introduction

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Medicinal plants produce various secondary metabolites as a part of their chemical defence and survival in nature [1]. These compounds have a wide range of biological activities and are widely used by humans. In agriculture, different medicinal plants have been studied for their allelopathic potential due to increasing public concern against synthetic agrochemicals [2, 3, 4]. It is known that herbs exert various physiological effects [5, 6]. Many secondary metabolites are used in contemporary medicine as drug ingredients [7]. Especially, researches are focused on potential of medicinal plants as a source of new effective anticancer agents [8, 9, 10].

Evaluation of antimitotic effect of plant extracts on *Allium* root tips is a part of preliminary anticancer studies [11]. Positive results disclose the potential to be used in cancer therapy [12]. Our previous studies revealed growth inhibitory activity of water extracts of *Origanum vulgare* ssp. *vulgare* L. and *Nepeta nuda* subsp. *nuda* (Lamiaceae) and *Adonis vernalis* L. (Ranunculaceae) on plant test system [13, 14, 15]. Present study aimed to evaluate the effect of the same extracts *in vitro* on proliferation/viability of human hepatoma cell line SK-HEP-1 *in vitro*.

Materials and methods

Plant material. *Adonis vernalis* and *Origanum vulgare* ssp. *vulgare* were collected in the vicinity of Shumen and *Nepeta nuda* subsp. *nuda* – near Sofia (Bulgaria). The plant specimens were identified and authenticated by Zheni Stoyanova (Taxonomist), Faculty of Natural Sciences, Shumen University, Bulgaria. Collected plant materials were dried at room temperature.

In our previous studies allelopathic effects of adonis, oregano and nepeta has been studied [13, 14, 15]. Based on our results, in present study plant solutions were prepared at following concentrations: *Adonis vernalis* – 1.83 mg/ml, *Origanum vulgare* ssp. *vulgare* – 3.5 mg/ml and *Nepeta nuda* subsp. *nuda* – 6.59 mg/ml.

Chemicals and reagents. MEM growth media and fetal calf serum were purchased from Sigma-Aldrich. Premixed WST-1 Cell Proliferation Reagent was purchased from Roche Applied Science.

Cell lines and culture conditions. The SK-HEP-1 was obtained from National Bank for industrial Microorganisms and Cell Cultures (Bulgaria). The cells were maintained as adherent in controlled environment: MEM medium, supplemented by 10% heat-inactivated fetal calf serum, in incubator at 37 °C, 5% CO₂ and humidified atmosphere. In order to keep cells in log phase, the cultures were refed with fresh medium two or three times/week.

***In vitro* cytotoxicity assay (dose-response relationship).** Cell proliferation/viability was assessed using Premixed WST-1 Cell Proliferation Reagent. The assay principle is based upon the reduction of the tetrazolium salt WST-1 to formazan in the mitochondria of living cells. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µl/well) at a density of 1×10⁵ cells per ml. Time of treatment was 24 hours.

Test solutions were freshly prepared in complete medium and filtered using 0.22 µm filter. Four hours before the end of incubation time, cell proliferating reagent WST-1 (10 µl/well) was added to the culture media. Microplates were further incubated for 4 hours at 37 °C. The absorbance of formazan product was quantitated at 450 nm using an ELISA reader. The cell survival fractions were calculated as a percentage of the untreated control (untreated control = 100%).

Data processing and statistics. The cytotoxicity assays were carried out in 9 replicate wells. The values for each concentration tested represent the average. Student's t-test was performed with $P \leq 0.05$ taken as a significance level.

Results and discussion

Plants used various chemical compounds to suppress growth and development of other plants. Some secondary metabolites have shown promising anticancer activity [10, 16, 17, 18]. Based on these data, in present study growth inhibitory effect of water extracts of three allelopathic plants on cancer cells *in vitro* was evaluated (Figure 1). As can be seen, adonis significantly inhibited vitality of SK-HEP-1. Oregano also exerted negative effect, but to a lesser degree. On the contrary, nepeta had an opposite effect, stimulating cell proliferation.

Figure 1. Effect of water extracts of *Adonis vernalis* (1.83 mg/ml), *Origanum vulgare ssp. vulgare* (3.5 mg/ml) and *Nepeta nuda subsp. nuda* (6.59 mg/ml) on proliferation/viability of human hepatoma cell line SK-HEP-1. ** $P \leq 0.01$; *** $P \leq 0.001$.

Recently secondary metabolites in medicinal plants are widely studied as potential anticancer agents. Cell viability assay is a useful tool in such studies. In present study adonis water extract had notable negative influence on cancer cell line tested. Cardiac glycosides have been discussed as cytostatics with possible applications in medicine [19]. Obviously, oregano also contains growth inhibitory compounds, but their influence was much weaker than adonis.

Interestingly, nepeta water extract stimulated cell viability. One possible explanation could be the type of extraction. Medicinal plants comprise source of various bioactive compounds. These compounds belong to different chemical families and have different solubilities. So, testing of medicinal plants requires a proper selection of extraction method [20]. Really, studies reveal that biological activity of plant extracts depends on solvents used [21, 22]. For example, according to [5] water extract of *Stevia rebaudiana* has no effect on HEP2 cells, but acetone and ethyl acetate extracts exerted cytotoxic effect. Taking into account the above-mentioned, in present study we compared the effect of nepeta water extract with influence of methanol extract. After treatment with nepeta methanol extract cell viability was significantly reduced (Figure 2).

Figure 2. Effect of water and methanol extracts of *Nepeta nuda* subsp. *nuda* (6.59 mg/ml) on proliferation/viability of human hepatoma cell line SK-HEP-1. *** $P \leq 0.001$

There are a lot of studies on plant extracts in order to find out novel effective anticancer drugs [23]. Principally, IC₅₀ values of extracts were compared with used in practice anticancer drugs. It must be noticed that in this preliminary study we used extracts at concentrations shown in previous studies to exert allelopathic activity. Secondary metabolites of plants are used as chemical weapon against other organisms. Humans have used secondary metabolites in traditional medicine since centuries. Nowadays their biological activity have been widely studied in order to find out new anticancer drugs. Present study contributes to characterization of biological activity of *Origanum vulgare* ssp. *vulgare*, *Adonis vernalis* and *Nepeta nuda* subsp. *nuda* and expands their potential use in medicine. Amongst three plant water extracts tested, only adonis showed significant inhibitory effect on cancer cells. Results revealed a lack of anticancer activity of nepeta water extract, but presence of promising bioactive compound in nepeta methanol extract. So, future researches should be focused on identification of metabolites with anticancer activity in adonis and nepeta.

Conclusion

The results of present study revealed that *Adonis vernalis* and *Nepeta nuda* subsp. *nuda* possess metabolites with growth inhibitory effect on human hepatoma cell line SK-HEP-1. All extract tested exerted activity at doses >1000 µg/ml. Further research is needed to clarify biological activity of lower concentrations which are appropriate to enable the design of new anticancer drugs.

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