



# Anticancer activity of *ex vitro* established *Achillea thracica* Velen. water extracts

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## ABSTRACT

*Ex situ* conservation of Bulgarian endemic species *Achillea thracica* offers a chance for development of a new antitumor drug. The aim of this study was to evaluate the anticancer effects of water extracts of *ex vitro* established *A. thracica* grown in Bulgaria against fibrosarcoma cells. In our preliminary study, cytotoxicity of hot and cold water extracts was determined using *Triticum* root elongation test and *Allium cepa* assay. *In vitro* cytotoxicity and pro-apoptotic activity of hot extract (HE) were estimated using HT1080 cell line fibrosarcoma cells. Cytotoxicity was determined by the change in the cell number, the cell viability, and the cell population growth. Pro-apoptotic activity was assessed by the score of cell nuclei with morphological changes. The result of the root elongation test showed that the HEs exerted a stronger inhibitory effect in comparison with the cold extracts (CEs). Respectively, EC50 value of the hot water extract is 9.85 g/l and of the CE – 12.71 g/l. The extracts tested at concentration equal to ½ EC50 and EC50 values showed a significant mitodepressive effect on *A. cepa* meristematic cells. The *in vitro* tests revealed a significant negative impact on cancer cell viability and a promising pro-apoptotic activity of hot water extract.

## INTRODUCTION

For centuries, the species of the genus *Achillea* (Asteraceae) have been used in traditional medicine. Phytochemical investigations have revealed that *Achillea* species contain a broad range of highly bioactive compounds [1]. In medicine, current studies are focused on secondary metabolites as a source of new effective anticancer agents [2–4].

The plant assays are used for preliminary screening of the plant extracts for their cytotoxic activity: *Allium cepa* root tip bioassay is an adequate system for assessing mitotic repressant activity [5]. *In vitro* studies using different cancer cell lines are useful tools to investigate the mechanism of plants' extracts cytotoxic action [6–9]. Cytotoxic and pro-apoptotic effects of water extracts of *Achillea* species *in vitro* have been reported [10]. Nowadays, medicinal plants' chemical compounds are largely explored by humans. They are based on the therapeutic use of plants

by indigenous people. Traditional medicines usually are prepared as whole water extracts of plants. Current studies have established different biological activity of cold and hot water extracts [11,12].

*Achillea thracica* is a critically endangered Bulgarian endemic species [13]. The aerial parts of *A. thracica* contain numerous secondary metabolites [14]. In modern society, a lot of valuable plant species are at risk of extinction. *Ex situ* conservation through the use of *in vitro* cultures may preserve the threatened plants [15,16]. In order to preserve *A. thracica*, a team from Sofia University provided a system for *ex situ* conservation through *in vitro* cultivation and *ex vitro* adaptation [17]. The chemical composition of plants depends on different factors. However, the constituents and properties of propagated plants should be studied.

The aim of this study was to evaluate the anticancer effects of water extracts of *ex vitro* established *A. thracica* Velen. grown in Bulgaria against fibrosarcoma cells.

## MATERIALS AND METHODS

### Water extracts

*Ex vitro* established plants were grown in the experimental field in Lozen mountain, Bulgaria [18]. Aerial

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parts of plants, cut about 30 cm from the top, were collected during the flowering period and dried at room temperature. Water extracts were prepared: 1) Hot extract (HE) - the air-dried and finely ground aerial parts were covered with boiling distilled water for 60 minutes. 2) Cold extract (CE) - the air-dried and finely ground aerial parts were placed in distilled water and left to stay for 24 hours at room temperature. Then, the aqueous extracts were filtered and evaporated at 50°C to obtain a dry extract.

#### Root elongation assay

The HEs were prepared at concentrations 8, 10, 12, 14, 16, 18 and 20 g/l, and the CEs - at concentrations 5, 10, 15, 20, 25 and 30 g/l using the corresponding dry extract. Seeds of *Triticum aestivum* L. were used. Five ml of each extract or distilled water as a control were applied to the seeds. The dishes were sealed and incubated at 25°C ± 1°C for 72 hours. The length of the roots of germinated seeds was measured. The percentage of root growth inhibition in relation to the control for each extract was determined. From the root growth curve, EC50 values were obtained: the effective concentration that decreased root growth about 50% when compared to the negative control group (distilled water, 100%) [19].

#### *Allium cepa* L.-test

Potential cytotoxicity and genotoxicity of water extracts were estimated using *A. cepa* L. (2n = 16) as a test object. The bulbs were kept for root germination in distilled water for 24 hours. Bulbs with new roots with a length of 1.5 cm were treated with HE and CE at concentrations ½ EC50 and EC50 of corresponding dry extract. Treatments were for 24 hours at 25°C ± 1°C. Temporary slide preparations were prepared [19]. The microscopic analysis included assessment of the mitotic index and aberrant cells.

#### *In vitro* cytotoxicity and pro-apoptotic activity of HE of *A. thracica*

##### Cell lines and culture conditions

The adherent HT1080 cell line fibrosarcoma cells were maintained in controlled environment: Dulbecco's Modified Eagle Medium, supplemented by 10% Fetal calf serum at 37°C and 5% CO<sub>2</sub>.

##### Test-object and treatment

HT1080 cells were treated with dry extract from HE at concentrations of 50, 100, 200, 400 and 600 µg/ml for 48 hours. 5-Fluorouracil at a concentration of 50 µg/ml was used as a positive control. After treatment the cells were harvested from cell culture, and *in vitro* cytotoxicity and pro-apoptotic activity of *A. thracica* HE were evaluated.

##### *In vitro* cytotoxicity

Cells were stained with trypan blue dye and analyzed in a Bürker chamber. The cytotoxic effect of the extracts was evaluated by the change in the total number of cells, viability, and population growth rate [20]. The growth rate constant was calculated as follows:

$$\kappa = \frac{(\ln N - \ln N_0)}{t}, \text{ where}$$

$\kappa$  - growth rate constant;

$N_0$  - number of cells at the start of the treatment;

$N$  - number of cells after the treatment;

$T$  - time of treatment.

#### Pro-apoptotic activity

HE-treated cells were harvested and fixed in Clarke's fixative. The cell suspension was spread on a microscope slide and after drying was stained with Giemsa dye. Microscopic preparations were examined for the presence of cells undergoing apoptosis. As indicators of apoptosis, cell nuclei with morphological changes were used - pyknosis (irreversible condensation of chromatin causing the nuclei to decrease in size), karyorrhexis (destructive fragmentation of a pyknotic nucleus) and karyolysis (nuclear fading caused by the dissolution of chromatin) [21].

#### Data processing and statistics

Student's *t*-test was performed with  $p \leq 0.05$  taken as a significance level.

## RESULTS AND DISCUSSION

#### Influence on root growth of *Triticum aestivum* L.

The potential antimetabolic effect of *ex vitro* established *A. thracica* on root growth of *T. aestivum* was evaluated. There are data about differences between extracts obtained at different temperatures [22]. Based on these observations, in the present study, we used HE and CE.

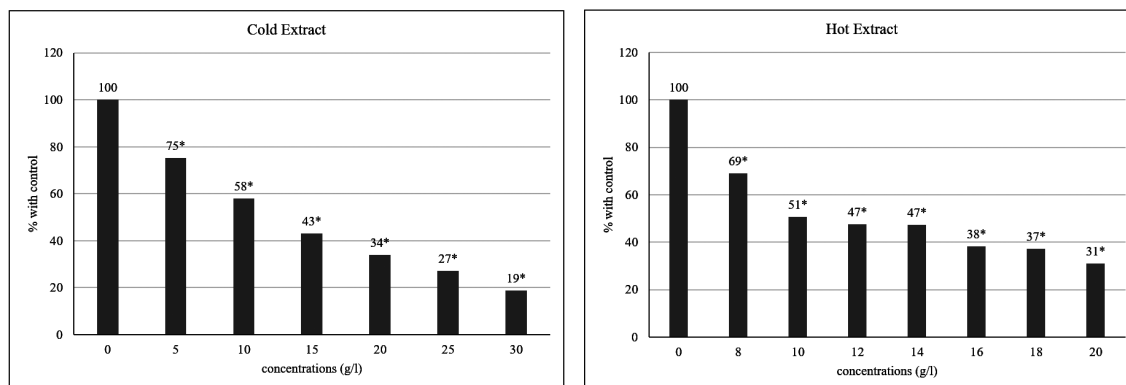
The results showed that both extracts caused similar effects on root growth (Fig. 1). An inhibitory effect on root growth as compared with controls was observed. Other studies also noticed that root elongation is a sensitive growth marker [23,24].

The Effective Concentration for 50% growth inhibition was estimated: EC50 = the effective concentration that decreased root growth by about 50% when compared to the negative control group [25,26]. EC50 value represents not only the degree of influence of extracts, but also usually is the first step in cytogenetic study of medicinal plants [27–29]. As can be seen from Figure 1, the HE exerted a stronger inhibitory effect on root growth in comparison with the CE. Respectively, the EC50 value of the hot water extract is 9.85 g/l and of the CE - 12.71 g/l. It can be speculated that the two extracts tested contain different bioactive compounds and/or different concentrations.

#### *Allium cepa* L.-test

Cytotoxic compounds could disturb the cell division [30]. Potential cytotoxicity of plant extracts was tested at concentration equal to ½ EC50 using *A. cepa*-test. The extracts tested showed a significant mitodepressive effect - the mitotic indices were extremely low (Table 1). In such a case it is not recommended to score chromosomal aberrations [26].

The decline of the mitotic index indicates the occurrence of a cytotoxic effect [31]. The findings of this study



**Figure 1.** Effect of *A. thracica* water extracts on root length of *T. aestivum* L. seeds. \* $p \leq 0.05$ .

showed the possibility of use *ex vitro* adapted *A. thracica* as an anticancer drug.

#### ***In vitro* cytotoxicity and pro-apoptotic activity of HE of *ex vitro* established *A. thracica* on human fibrosarcoma cell line HT 1080**

It is well known that conventional medicine uses different plant-derived compounds to treat cancer [24]. The secondary metabolites in medicinal plants are widely studied as new potential anticancer agents. Cytotoxic effects of different *Achillea* species have been reported [10].

Evaluation of the antimetabolic effect of plant extracts on *Allium root* tips is a part of preliminary anticancer studies [5]. Positive results disclose the possibility to be used as an antineoplastic drug [32]. *In vitro* cell test systems are widely used to establish the specific properties of chemical compounds [33]. The human cell lines of pathogenic origin offer the opportunity to evaluate the therapeutic effectiveness of potential new drugs directly on cancer cells [34]. The results of the phytotoxicity test of *A. thracica* water extracts revealed stronger growth inhibitory effect of the HE. However, we tested the possible cytotoxicity of the HE on cancer cells *in vitro*. In the present study, human fibrosarcoma cell line HT 1080 was used as a test-object.

#### ***In vitro* cytotoxicity effect on the cell population**

The results of the treatment with HE of *A. thracica* on the total cell number, the cell viability and the population growth rate are summarized in Table 2. The cell number was reduced to a great degree after treatment with extracts at concentrations of 400 and 600  $\mu\text{g/ml}$  - respectively 2.7 and 4.8 fold as compared to the untreated control. The extracts tested significantly inhibited the proliferation/viability of cells. The growth rate constant was lowered. The control cell population increased by 8.18% every hour. After treatment with extracts at concentrations of 400 and 600  $\mu\text{g/ml}$ , this value was respectively 6.09% and 4.91% hourly.

#### ***Pro-apoptotic activity***

The cancer cells can be eliminated by induction of apoptosis [35]. The morphological changes in the cell nucleus

**Table 1.** Effect of treatment with *A. thracica* water extracts at concentrations  $\frac{1}{2}$  EC<sub>50</sub> and EC<sub>50</sub> (for 24 hours) on root tip meristematic cells of *A. cepa* L.

Sample	Mitotic index		Abnormal cells	
	Mean	(% $\pm$ SD)	Mean	(% $\pm$ SD)
Control	4.81	$\pm 0.71$	5.10	$\pm 3.82$
CE				
$\frac{1}{2}$ EC <sub>50</sub> (6.35 g/l)	<1.00*	–	–	–
EC <sub>50</sub> (12.71 g/l)	<1.00*	–	–	–
HE				
$\frac{1}{2}$ EC <sub>50</sub> (4.93 g/l)	<1.00*	–	–	–
EC <sub>50</sub> (9.85 g/l)	<1.00*	–	–	–

Data are presented as mean  $\pm$  SD; \* $p \leq 0.05$ .

**Table 2.** *In vitro* cytotoxicity effect of treatment with *A. thracica* hot water extract (for 48 hours) on human fibrosarcoma cell line HT 1080.

Dose ( $\mu\text{g/ml}$ )	Total number of cells ( $n \times 10^5$ )		Viability (%)		Growth rate constant (%/hour)	
	mean	$\pm$ SD	mean	$\pm$ SD	mean	$\pm$ SD
0	50.96	$\pm 4.85$	97.77	$\pm 0.75$	8.18	$\pm 0.196$
50	51.00	$\pm 4.04$	96.01*	$\pm 0.96$	8.19	$\pm 0.159$
100	58.32*	$\pm 3.94$	97.11*	$\pm 1.36$	8.47*	$\pm 0.143$
200	46.44*	$\pm 2.48$	96.10*	$\pm 1.90$	7.99*	$\pm 0.112$
400	18.68*	$\pm 2.24$	95.62*	$\pm 1.47$	6.09*	$\pm 0.266$
600	10.60*	$\pm 1.13$	70.99*	$\pm 7.04$	4.91*	$\pm 0.216$
50 <sup>#</sup>	9.64*	$\pm 1.28$	89.85*	$\pm 2.93$	4.71*	$\pm 0.283$

Data are presented as mean  $\pm$  SD; #—positive control (5-Fluorouracil); \* $p \leq 0.05$ .

serve as signs of apoptosis [36–38]. The ability of studied extracts to induce apoptosis was assessed by the evaluation of nuclear morphology changes in Giemsa-stained cells. Three stages of the nucleus at the early stage of apoptosis were established - pyknosis, karyorrhexis and karyolysis.

**Table 3.** Proapoptotic effect of treatment with *A. thracica* hot water extract (for 48 hours) on human fibrosarcoma cell line HT 1080.

Dose (µg/ml)	Analised cells	Nuclear morphology changes			Total (% ± SD)
		Pyknosis	Karyorrhexis	Karyolysis	
0	613	26	6	0	5.37 ± 1.13
50	688	114	3	2	17.30 ± 2.16*
100	696	89	0	22	15.95 ± 1.89*
200	669	158	0	22	23.62 ± 1.75*
400	656	98	2	56	23.78 ± 1.92*
600	615	86	2	112	32.52 ± 2.34*
50 <sup>#</sup>	639	21	0	3	3.69 ± 0.48

Data are presented as mean ± SD; #—positive control (5-Fluorouracil); \* $p \leq 0.05$ .

Treatment with extracts increased significantly (three- to fivefold) the percent of the cells with nuclear morphology changes in comparison with the negative and the positive control (Table 3). It should be noted that pyknosis was established after treatment with all extracts. Treated with extract cells revealed also a sign of karyorrhexis. The nucleus alterations could be a reason for these results [38]. Karyolysis have been observed only in the treated cells and in the positive control. The frequency of induction of karyolysis is dose-dependent.

Many antitumor drugs are known to be effective only against certain tumor cells. Therefore, the first step in the investigation of new antitumor drugs is to establish their activity against tumor cells of different cell lines [39]. In the present study, hot water extract of *A. thracica* showed cytotoxic and pro-apoptotic effects on human fibrosarcoma cell line HT1080.

## CONCLUSION

*Achillea thracica* hot and cold water extracts have cytotoxic effect. The HE showed a stronger inhibitory effect on root growth in comparison with the CE. Both extracts exerted mitodepressive effect on *A. cepa* meristematic cells at concentrations equal to ½ EC50 and EC50 values. *Achillea thracica* hot water extract exerted a significant dose-dependant negative impact on HT1080 cell viability and showed promising pro-apoptotic activity.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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