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Allelopathy of Cold Water Extracts from *Origanum vulgare* ssp. *vulgare* L.

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Abstract

Secondary metabolites in medicinal plants could lead to discovery of new classes of herbicides. Recently aromatic plants have gained interest as a source of allelopathic secondary metabolites. *Origanum vulgare* ssp. *vulgare* L. infusions in hot water are used in folk medicine and possess proved beneficial biological activity. Plant-to-plant variability of metabolites due to genetic heterogeneity is established in Lamiaceae family. From this point of view, studies on plants from different geographic regions might reveal important sources of variability. The objective of this study was to evaluate allelopathic activity of cold water extracts made from the aerial parts of *O. vulgare* ssp. *vulgare* growing wild in Northeast Bulgaria in laboratory conditions. The allelopathic effect was evaluated using root elongation test and *Allium cepa*-test. Oregano extracts (17.5 g/l, 52.5 g/l) significantly decreased root length of *Triticum aestivum* L. ($P \leq 0.001$). The root growth reduction could serve as a sign for presence of water soluble allelopathic secondary metabolites in the plant tested. Oregano (3.5 g/l) inhibited cell division in *Allium* root meristematic cells. The decline of the mitotic index indicates the occurrence of a cytotoxic effect. Oregano induced abnormalities in mitotic and interphase cells, so can be also considered as genotoxic. The observed macroscopic and microscopic effects of tested extracts indicated presence of water soluble allelochemicals in *O. vulgare* ssp. *vulgare*. This characteristic could be further studied as a possibility to be used in weed management programs.

Keywords

***Origanum vulgare* ssp. *vulgare* L., Allelopathy, Root Growth Inhibition, *Allium cepa*-Test**

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1. Introduction

Oregano (Lamiaceae) is a valuable aromatic plant found in natural conditions in Europe, Central Asia and North America [1]. One of the most commonly used subspecies is *Origanum vulgare* ssp. *vulgare* L. [2]. This plant occurs naturally in Bulgaria. Recently aromatic plants have gained interest as a source of allelopathic secondary metabolites. Allelopathy as a phenomenon where a plant chemically affects other plants has been known for centuries. The renewed interest in allelopathy to a great extent is due to the necessity to reduce synthetic chemical input into agriculture [3] [4]. According to [5] aromatic plants, such as oregano, could be used in agriculture for the suppression of some weeds. Allelochemicals could cause inhibition of seed germination and/or seedling growth [6]. A number of studies reported that germination was less sensitive than seedling growth [7]-[9]. According to [10] the inhibition of root growth and development by allelochemicals can be due to changes in DNA synthesis in cells of apical root meristem, alteration of the mitochondrial metabolism [11] or changes in cell mitotic indices [12]-[15].

Allelopathic activity of essential oils has been widely studied [16]-[18]. There are also data about water-soluble phytotoxic compounds in aromatic plants [5] [6]. Oregano hot water infusions are used in folk medicine and possess proved biological activity. According to data about different effect of extracts obtained at different temperature [19] [20], a matter of interest is evaluation of the biological activity of cold water extracts. Following secondary metabolites have been identified in water extract of *O. vulgare* ssp. *vulgare*: glicosides, coumarins, phenolic compounds and tannins, flavanonols, anthocyanins [21]. Some of these compounds have been reported as potent allelochemicals: phenolic compounds, namely rosemarinic acid, chlorogenic acid, caffeic acid [22]-[24]. It must be noticed that the bioactivities of crude extracts are often different to those seen for the individual components: crude plant extracts may contain different chemical constituents that interact in complex ways [25].

The objective of this study was to evaluate allelopathic activity of cold water extracts made from the aerial parts of *Origanum vulgare* ssp. *vulgare* growing wild in Northeast Bulgaria in laboratory conditions.

2. Materials and Methods

2.1. Plant Materials

Origanum vulgare ssp. *vulgare* growing wild in the vicinity of Shumen (Velino, Bulgaria) (latitude 43˚18'N; longitude 27˚01'E, altitude 227 m) was used in this study. The aerial parts of oregano plants during the flowering stage were collected in June-July. The plant specimens were identified and authenticated by Nanova (Taxonomist), Faculty of Natural Sciences, Shumen University, Bulgaria. Collected plant materials were dried at a room temperature.

2.2. Plant Water Extracts

Aerial parts of oregano plants, cut about 20 cm from the top were used. The dried oregano tissue was placed in distilled water and left to stay for 24 h at room temperature. Oregano Cold Water Extracts (OCWE) were prepared at concentrations 3.5 g/l, 17.5 g/l (5× more concentrated) and 52.5 g/l (15× more concentrated). The extracts were used after filtration.

2.3. Root Elongation Test

Twenty seeds of *Triticum aestivum* cv. GTW were placed on filter paper in each of three Petri dishes (11 cm in diameter). Five ml of OCWE (17.5 g/l and 52.5 g/l) or distilled water, as a control, was applied to the seeds. The dishes were sealed and incubated at 25˚C ± 1˚C for 72 h. The length of the roots of germinated seeds was measured. The percentage root growth inhibition in relation to the negative control for each extract was determined. Seeds that did not germinate were not included in the root elongation test. Three replications of each treatment were done.

2.4. *Allium cepa*-Test

Microscopic cytotoxic effects of OCWE were evaluated using *Allium cepa*-test [26]. Thirty seeds of *A. cepa* were placed on filter paper in each of three Petri dishes (11 cm in diameter), containing 5 ml of distilled water.

The Petri dishes were sealed and incubated at 25˚C ± 1˚C for 48 h. Twenty germinated seeds with equal length of roots (~1 cm) were removed and placed on filter paper in each of another three Petri dishes. Five ml of OCWE (3.5 g/l) were added to one of the dishes, and incubated at 25˚C ± 1˚C for 3 h. Distilled water was used as a negative control and methyl methanesulfonate (11 mg/l, for 24 h) was used as a positive control. After treatment, the roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 min, hydrolysed in 3 N HCl for 8 min and in 45% acetic acid (CH_3COOH) for 30 min at room temperature and stained for 40 min in 2% acetoorseine. After staining, the terminal root tips (1 - 2 mm) were cut off and squashed in 45% CH_3COOH. The microscopic analysis included the mitotic index and scoring of aberrant cells. Each sample consisted of six root meristems. At least 1000 cells of each root meristem were analysed. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of analysed cells. The index of each phase of mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells.

2.5. Statistical Analyses

We processed the experimental data by Student's *t*-test. In germination and root inhibition test we choose as an experimental unit the root. The calculations were carried out on the assumption that roots used in each treatment made one sample, and each sample was tested against the control sample. In *Allium cepa*-test we choose as an experimental unit the cell, instead of the root. The calculations were carried out on the assumption that all the cells of the six root meristems made one sample, and each sample was tested against the negative control.

3. Results and Discussion

3.1. Root Elongation Test

In present study root growth inhibition of *T. aestivum* was used as index of general toxicity [27] [28]. Economou *et al*. [20] tested allelopathic influence of Greek oregano at concentrations in the 18.75 - 150 g/l range. Keeping these data in view we tested OCWE at concentrations 17.5 g/l and 52.5 g/l.

Data on growth inhibition effect of OCWE are presented in **Table 1**. As can be seen, both concentrations tested significantly inhibited root elongation of *T. aestivum*. This negative effect is dose dependent. The established root growth reduction could serve as a sign for presence of water soluble allelopathic secondary metabolites in the plant tested. This observation is in accordance to data that medicinal plants could possess allelopathic activity [6].

3.2. *Allium cepa*-Test

The macroscopic effect of plant extracts could be confirmed by microscopic studies providing definitive information regarding the extent of cytotoxic action [29]. We evaluated microscopic cytotoxic effects of OCWE using *Allium cepa*-test. The results of a preliminary experiment (data not shown) revealed a strong mitodepressive effect of OCWE at concentration of 17.5 g/l. It is not recommended to provide cytogenetic analysis if the mitotic index is very low [26]. So we provided *Allium cepa*-test using the concentration 3.5 g/l, normally used by population [30]. Treatment time of 3 h was used since this period corresponded to the earliest appearance of DNA damage [31] [32].

Table 1. Effect of OCWE on root elongation of *Triticum aestivum* L., germinated in the infusions over a period of 72 h.

Sample	Concentrations			
	17.5 g/l		52.5 g/l	
	Root length, mm ± SD	% compared to the control	Root length, mm ± SD	% compared to the control
Control	55.72 ± 12.02	100	55.72 ± 12.02	100
OCWE	33.29 ± 7.35	59.75***	16.33 ± 4.26	29.31***

Data are expressed as means from three replications done ± SD (standard deviation), ***P ≤ 0.001, OCWE-Oregano Cold Water Extract, Control-distilled water.

Table 2 summarizes the effect of OCWE on mitotic index and mitotic phase in the root meristematic cells of *A. cepa*. Upon the treatment the mitotic index was decreased in comparison with the negative control. Allelochemicals can affect the plant growth by different manner: one of the principal mechanisms is the alteration of the mitotic index [33]. The decline of the mitotic index after treatment with OCWE indicates the occurrence of a cytotoxic effect [34]. The treatment also changed the mitotic phase distribution. The characteristic effect caused by OCWE was an increase of anaphase index and simultaneous decrease of telophase index. The interference in the cell cycle kinetics also is accepted as a sign of cytotoxic influence [35].

Table 3 shows the frequency and the types of abnormalities observed upon exposure of root tips of *A. cepa* to OCWE. The treatment with oregano significantly increased the percent of chromosome aberrations in comparison to negative control. Disturbed metaphases and anaphases (**Figure 1(A)** and **Figure 1(B)**) are the most frequently observed abnormality noted in the treated cells. Bridges and fragments in ana-telophase were the second (**Figure 1(C)** and **Figure 1(E)**). Laggard and vagrant chromosomes were also scored (**Figure 1(D)**). Disturbed metaphases and anaphases may be caused by inhibition of spindle formation [36] [37]. Laggard chromosomes also indicate spindle disturbances [26]. Fragments and bridges may be consequence of DNA breaks [38]. According to [34] chromosome bridges and breaks in mitotic cells are indicators of a clastogenic action. As can be

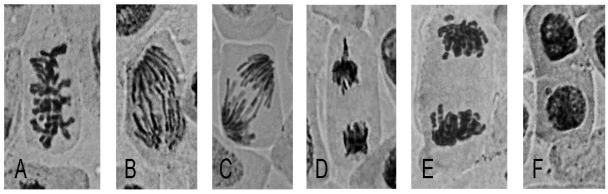

Figure 1. Aberrations induced by OCWE in *Allium cepa* root tips: (A) Abnormal metaphase; (B) Spindle abnormalities in anaphase; (C) Bridge; (D) Laggard chromosome in anaphase-telophase; (E) Anaphase-telophase with fragment; (F) Micronucleus in interphase cell.

Table 2. Effects of treatment with OCWE (3.5 g/l, for 3 h) on mitotic index and phase indices in root tip meristematic cells of *Allium cepa* L.

Sample	Number of cells analysed	Number of dividing cells	MI% (±SD)	Prophase PhI% (±SD)	Metaphase PhI% (±SD)	Anaphase PhI% (±SD)	Telophase PhI% (±SD)
NC	8895	510	5.73 (±0.23)	24.90 (±0.43)	26.47 (±0.44)	18.82 (±0.39)	29.80 (±0.46)
OCWE	10736	538	5.01 (±0.22)*	25.46 (±0.44)	26.39 (±0.44)	23.98 (±0.42)*	24.16 (±0.43)*
PC	6982	251	3.59 (±0.19)***	21.12 (±0.41)	30.68 (±0.46)	25.50 (±0.44)*	22.71 (±0.42)*

Sample: NC: negative control (distilled water); PC: positive control (methyl methanesulfonate, 11 mg/l); OCWE: Oregano Cold Water Extracts. MI%: Mitotic Index (%); PhI%: Phase Index (%). Data are expressed as means ± SD (standard deviation), $^{*}P \leq 0.05$, $^{***}P \leq 0.001$.

Table 3. Mitotic abnormalities and interphase cells with micronuclei in root tips of *Allium cepa* L. after treatment with OCWE (3.5 g/l, for 3 h).

Sample	Abnormalities, % of mitotic cells				Total abnormalities in mitotic cells, % ± SD	Micronuclei in interphase cells, % ± SD
	Spindle abnormalities in metaphase	Spindle abnormalities in ana-telophase	Bridges and fragments in ana-telophase	Laggard and vagrant chromosomes		
NC	-	1.57	0.39	0.20	2.16 ± 0.15	0.12 ± 0.04
OCWE	1.12	1.49	1.49	0.93	5.02 ± 0.23*	0.27 ± 0.05*
PC	0.40	-	11.96	4.38	16.73 ± 0.37***	0.47 ± 0.07***

Data are expressed as means ± SD (standard deviation), $^{*}P \leq 0.05$, $^{***}P \leq 0.001$; Sample: NC: negative control (distilled water); PC: positive control (methyl methanesulfonate, 11 mg/l); OCWE: Oregano Cold Water Extract.

seen from **Table 3**, the most frequent aberrations were associated with spindle dysfunction (spindle abnormalities in metaphase and ana-telophase, laggard and vagrant chromosomes) rather than clastogenicity (bridges and fragments).

Additionally, we observed elevated percent of micronuclei in interphase cells in treated group as compared to those exposed to water only (**Table 3**). Micronuclei are extranuclear bodies of chromatin material (**Figure 1(F)**). These bodies result from damages, not or wrongly repaired, in the parental cells [34]. The frequency of cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals [38].

Data in **Table 3** indicated that in addition to the cytotoxicity, OCWE can be also considered as genotoxic. These potentialities play important role in agroecosystems since the allelochemicals produced by plants can be utilized to protect the environment [27]. Application of allelopathic plant extracts can effectively control pests [39] [40] and identified natural products may lead to discovery of new herbicides [41].

The results of root elongation test and *Allium cepa*-test revealed allelopathic activity of *O. vulgare* ssp. *vulgare* growing wild in Northeast Bulgaria. Plant-to-plant variability of metabolites due to genetic heterogeneity is established in Lamiaceae family [42] [43]. Environmental factors and the stage of growth also influence Origanum composition [1]. From this point of view studies on plants from different geographic regions could reveal important sources of variability.

4. Conclusion

The observed macroscopic and microscopic effects of OCWE indicated presence of water soluble allelochemicals in *O. vulgare* ssp. *vulgare* growing wild in Northeast Bulgaria. This characteristic could be further studied as a possibility to be used in weed management programs.

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