



Effect of The Water Origin on The Biological Properties of Sage (*Salvia officinalis* L.) Aqueous Extracts

Irina Korotkova*, Tamila Romashko, Oleg Khakhel', Tamila Zvenihorodska, Tetiana S Yaprynets, and Viktor Liashenko

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Abstract

Plant extracts, due to the high content of various biologically active compounds, have found their wide application in the foods, pharmaceutical industries, and agricultures, for the production of valuable and useful bioproducts. The aqueous plant extracts are most often used. The effect of the water origin used in their preparation on the extract's composition has not been studied enough, which limits their use. The study was aimed to compare the biological effect of sage (*Salvia officinalis* L.) extracts prepared using water of different origins (distilled, tap, spring, bottled). The preparation of aqueous extracts was carried out in two ways, differing in the extraction temperature. In the first method, boiling water was used to prepare the extract; in the second, cooled boiled water was used. The sage leaves mass for extract preparation was 1.65 and 3.30 g. The biological activity of the extracts was assessed by testing them for radish germination. It was shown the sage leaves extracts prepared using various waters origin can differ by one and half times in their allelopathic effect on radish seeds. It was proved the qualitative composition and mass of the substance extracted from the plant depend on the water origin used for extracts preparation. Thus, using the same amount of plant material to prepare extracts, you can increase their biological activity by fifty percent with the right choice of water.

Keywords: allelopathy, germination, *Raphanus sativus* L. var. *radicula* Pers., seeds, aqueous plant extracts, infusion, decoction

1. INTRODUCTION

Plant extracts have a long history of their study and use. Firstly, this concerns extracts of medicinal plants, which are used as a raw material base for the pharmaceutical industry. Now, numerous secondary metabolites with different structures and pharmacological properties have been identified in plants which has served as the basis for the development of phytopharmaceuticals. The plant extracts application has led to a new concept known as polypharmacology, where natural products from different plant sources can exert broad therapeutic effects [1][2].

Relatively recently, other areas of research on plant extracts have emerged, which aim to replace everywhere synthetic chemicals with their natural counterparts. So, plant extracts began to be widely used as an alternative to synthetic plant protection products and for biostimulants production [3][4].

Plant extracts with significant antibacterial activity against certain bacteria have found use as natural pesticides [5].

Plant extracts for food drinks and plant-based cosmetics are being developed [6]-[8]. Many plant extracts have antioxidant and antimicrobial properties and are widely used as nature additives in the food industry [9], antioxidants during food storage [10], a source of antimicrobials agents for biomedical applications [11], and metal corrosion inhibitors [12]-[14]. One of the research areas into plant extracts is associated with the "green" synthesis of metal nanoparticles and their oxides [15][16].

The unique biological properties of plant extracts are mostly determined by high content of biologically active compounds with a properly selected technology for extracting plant biomass [17]-[19]. In the classic extraction methods, the organic solvents are used because it's selectively extract target compounds. However, this method is quite labor-intensive and requires many solvents volumes. In addition, final products may contain trace amounts of solvent, which limits its usage [20].

In many applications, water is the predominant solvent for biomass extraction, despite the limited solubility of some bioactive substances in it [21]. In this regard, methods are being developed to enhance water potential as solvent to extract low-water solubility compounds [22]. Aqueous plant

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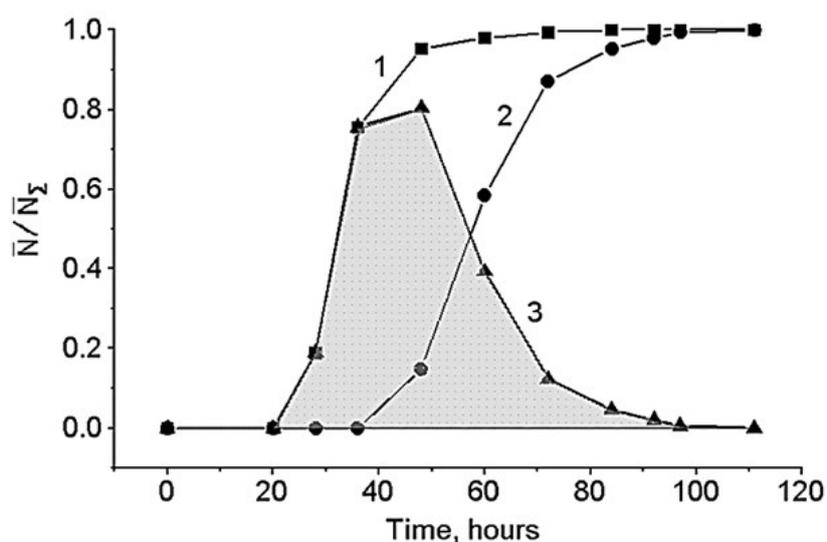


Figure 1. Dynamics of radish seed germination: curve 1 – in spring water (water 3), curve 2 – in an extract prepared using this water (decoction, sage leaves 3.30 g/100 mL of water); curve 3 is the difference of curves 1 and 2. \bar{N}/\bar{N}_z is the ratio of the seed's germinated number to all seeds.

extracts have a number of advantages, such as ecologically and non-toxic to animals and humans, and they are therefore widely used in medicine and food industry, for the preparation of drugs, plant stimulants and protection agents [23][24]. Particularly noteworthy are the methods of biological plant protection from weeds based on the phenomenon of allelopathy. These methods are based on the effect of some plants on others as a result of the metabolites production and release into the environment [25], which act as natural herbicides [26]-[28].

At present, the allelopathic effect has been established for a number of aqueous plants extracts. The concentration effect of aqueous extracts on the seed germination process has been studied. It was found that low extracts concentrations have a weak stimulating effect, while high concentrations inhibit seed germination. Szafraniec et al. showed that an aqueous solution of peppermint leaves (*Mentha piperita* L.) at a concentration of 1 g/L increased radish seeds germination, while doses of 5 and 10 g/L acted as inhibitors [29]. Zandi et al. also found that aqueous extracts of *Stellaria media* (L.) Vill. can stimulate or inhibit radish seed germination depending on the form of extract and the concentration of chemical compounds in extracts [30].

Distilled water is usually used to obtain plant extracts. However in industry, the use of distillate is

expensive, and it forces you to choose natural water. Natural mineral water composition depends on its origin, which raises the question of how this composition has effect on biological capabilities of extracts. However, there are not many researches devoted to this problem. The results of Ma et al. (2024) and Wyrostek and Kowalski (2021) studies indicate that waters with lower mineralization extract a greater amount of biologically active substances [31][32]. That is, the influence of water mineral composition on the extraction process efficiency and extracts biological activity has not been clearly established and requires further study. To find out, whether such an effect exists, in this study, plant extracts prepared using waters of different origins were compared, where their biological activity was assessed by the germination test with radish (*Raphanus sativus* L. var. *radicula* Pers.) seed.

2. MATERIALS AND METHODS

2.1. Materials

Sage leaves (*Salvia officinalis* L.) samples which purchased from a pharmacy (producer: PJSC "Liktravy", Ukraine) were used for the aqueous extracts' preparation. Radish seed (*Raphanus sativus* L. var. *radicula* Pers.) Zhuravlinà cultivar's of the Ukrainian selection were used in the experiment. Chemicals for the acid-base titration

were 0.05 M HCl solution, 0.1% methyl orange, 0.1% phenolphthaleinin solution in water-ethanol mixture (8:2 v/v), and 5% aqueous solution of sodium hypochlorite. All reagents and chemicals used in this study were of analytical grade.

2.2. Methods

2.2.1. Preparation of Aqueous Extract

Two experiments were carried out within the study, differing in the extraction temperature. In the first way of the extract preparation (decoction) according to the previous study [33], water was boiled, after which a sample of sage leaves (1.65 and 3.30 g) placed in a 250 mL conical flask, and then poured with boiling water (100 mL), followed by evaporation in a water bath for 15 min. The extracts were then cooled in a desiccator for 1 h. The second way (infusion) differed from the first one in the use of boiled cooled water [34]. The purpose of boiling was to eliminate possible bacterial water contamination. The amount of herb was taken the same as in the first way (1.65 and 3.30 g). These samples were poured with boiled cooled water (100 mL) and kept in a dark place at a temperature of 20 °C for 24 h. After extraction, all extracts were filtered through sterile gauze, squeezed, filtered through a sterilized filter paper and used immediately. Also at this stage, to determine the extracts dry rest, 50 mL of extracts

were taken, placed in weighed 100 mL flasks, evaporated on a water bath and dried for 3 h at 105 °C, then cooled and weighed.

2.2.2. Tested Waters

Experiments were carried out with distilled water (dist), as well as with three fresh water samples: tap water, bottled water and spring water. Tap water (water 1) is drinking water, which has an artesian origin from the Cenomanian-Lower Cretaceous horizon (depth 400–800 m). Bottled water (water 2) is non-carbonated water from the producer LLC “Akvaplast”, Ukraine. The type of initial water is surface water. This is lake or river water that has been purified to bring it into line with drinking water standards. Spring water (water 3) was taken from a natural spring located within the Poltava city, Ukraine. Data on the pH, total acidity and hydrogen carbonate ions content in tested waters and sage leave's aqueous extracts were shown in Table 1. Determination of pH was carried out using an I-160MI ionometer. The pH measurement error is 0.03 [35]. Hydrogen carbonate ions content in water sample was determined by acid-base titration in accordance with previous work [36].

2.2.3. Seed Germination Test

Germination tests were conducted with radish seeds (*Raphanus sativus* var. *radicula*) using three replications as per procedure described by ISTA

Table 1. pH, total acidity and hydrogen carbonate ions content of tested waters and sage leaves' aqueous extracts.

Extracts recipe	pH			
	dist	water 1	water 2	water 3
0.00 g/100 mL (pure waters before/after boiling)	5.16/6.15	8.46/8.79	7.33/8.78	7.09/7.63
1.65 g/100 mL (infusion/decoction)	5.30/6.19	5.83/7.56	5.55/7.04	6.19/7.56
3.30 g/100 mL (infusion/decoction)	5.47/5.66	5.66/7.18	5.72/6.53	6.06/7.02
Total acidity, μmol/L				
0.0 0 g/100 mL (pure waters before/after boiling)	-/23	4800/4650	2150/2000	2500/10150
Hydrogen carbonate ions, μmol/L				
0.0 0 g/100 mL (pure waters before/after boiling)	-/24	46507/4433	2131/1909	12570/10117

Note: data are shown as mean ± SD (n = 3), $p < 0.05$

Table 2. Descriptive statistics of a percent of total germinated seeds radish for tested waters.

Tested water	Germinated seeds (%)				
	Mean	Median	Min.	Max.	SD
dist	97.9	98	96	100	1.6
water 1	96.3	96	92	98	1.7
water 2	99.5	100	98	100	0.9
water 3	98.0	98	96	100	1.3

(2023) [37]. To germinate radish, petri dishes were lined with sterilized filter paper discs and 50 seeds of radish were placed into each one. Before that, the seeds were sterilized. A gauze bag with seeds was immersed in a 5% aqueous solution of sodium hypochlorite and kept under the UV lamp of a laminar box for 10 min. Then, the seeds were washed several times with distilled water. Into each dish, 3 mL of extract of appropriate concentration was added. The dishes were placed in a seed germination box at 22 °C in the dark. The control dishes consisted of seeds and pure waters. The seeds were considered germinated when a root length more than 5 mm was appeared. Such seeds were counted and removed from the dishes. Measurements were stopped 111 hours after the start of germination, after which ungerminated (dead) seeds were counted.

2.2.4. Data Analysis

In general, an extract's allelopathic effect on a test plant can lead to its death, delayed germination and inhibition of further development. In our experiment, the percentage of radish seed germination was determined, and the delay time in seed germination under the effect of sage leaves aqueous extracts was studied. The germination percentage was determined by the ratio of the total number of germinated seeds to the total number of seeds sown in petri dishes. This parameter was calculated as described by Onofri et al. [38]. The delay time for radish seeds germination was estimated by the S parameter proposed by us in this article. The calculation procedure of delay time of seed germination (S) is explained further in Figure 1.

As an example, Figure 1 illustrates curves featuring the dynamics of the radish seeds germination in pure water 3 (curve 1) and in an extract of sage leaves prepared using this water (curve 2). Dependencies 1 and 2 represent the magnitudes of \bar{N}/\bar{N}_z as a function of germination time. \bar{N} is the average number of germinated seeds for three repetitions at a certain time, and \bar{N}_z is the average number of all germinated seeds in the treatment. Dependencies 1 and 2 can be used to estimate the extract allelopathic effect. The difference between curves 1 and 2 is present by a curve 3 in Figure 1. The area under curve 3 has the dimension of time, and this is S in the test relative to the control. Descriptive statistics, testing of the null hypothesis and ANOVA were performed using the STATISTICA 10 software for Windows (Stat-Soft). Homogeneous groups were analyzed by Tukey's test ($p \leq 0.05$).

3. RESULTS AND DISCUSSIONS

3.1. pH Values, Total Acidity and Hydrogen Carbonate Ions Content in The Samples of The Experiment

The pH of the waters used in the experiment ranged from 7.09 (spring water) to 8.46 (tap water). After boiling, all waters were characterized by an increased pH value, which correlated with a decrease in the hydrogen carbonate ions content. The lowest pH value was recorded for spring water (7.63), while tap water and bottled water had approximately the same pH values (8.79). The obtained our results are consistent with the study by Ma et al., explaining this change by a decrease in CO₂ dissolved in water. pH of the obtained herbal

extracts prepared by infusion, regardless of concentration, was significantly lower than that of the extracts prepared by decoction. The lowest pH value was recorded for extracts with a concentration of 3.30 g/100 mL, prepared by both infusion and decoction methods [39].

3.2. Effect of The Water Origin on The Seed Germination

The number of germinated radish seeds in different waters origin is presented in Table 2. As can be seen, the percentage of germinated radish seeds is quite high for all waters, especially for water 2 (median = 100%; Table 2). Differences between waters exist, but they are small. The gap between the worst and the best result was about three per cent. Seed germination was most negatively affected by tap water (water 1) and positively affected by packaged water (water 2), as shown by the histogram in Figure 2.

From these data it is also seen that there is no significant difference between both extracts prepared by different ways and extracts with different concentrations. Calculations confirm a significant effect of water origin (ANOVA: $F(3,40) = 13.7$, $p < 0.0001$), competing with extracts concentration ($F(4,40) = 0.6$, $p = 0.7$) and the interaction between these factors ($F(12,40) = 1.4$, $p = 0.2$) on the seed death.

Considering, the number of germinated seeds is affected exclusively by the water origin; we can ignore the factors of the preparation way of extracts

and their concentration, that is, unify the data of different tests based on the water origin and perform statistical calculations with the larger sample size. The results of these calculations are illustrated in Figure 3. It was shown the spring water forms a homogeneous group with distilled water, while tap water worsens seed germination, and bottled water, on the contrary, somewhat reduces the percentage of dead seeds.

The action mechanism of the most allelochemicals is definitely not established at present. However, it is known that the allelopathic effect is individual and depends on the donor plant and the recipient plant [28]. That is this effect may be or may not be and obviously, here we have just the case when the effect of extracts on seed germination is absent. The differences observed in the experiment can be explained by the better or worse physiological equilibrium of a salt solution in different waters.

3.3 Effect of The Water Origin on The Delay Time of Seed Germination

The authors of many studies associate the bioactivity of sage extracts with phenols and flavonoids contained in them [39]-[41]. Although biologically active substances of sage turned out to be inert with regard to the effect on the germination of radish in our experiments, they significantly affected the delay in the timing of its germination. Test results proving this are shown in Figure 4.

For all treatments (Figure 4(a)-(d)) there are

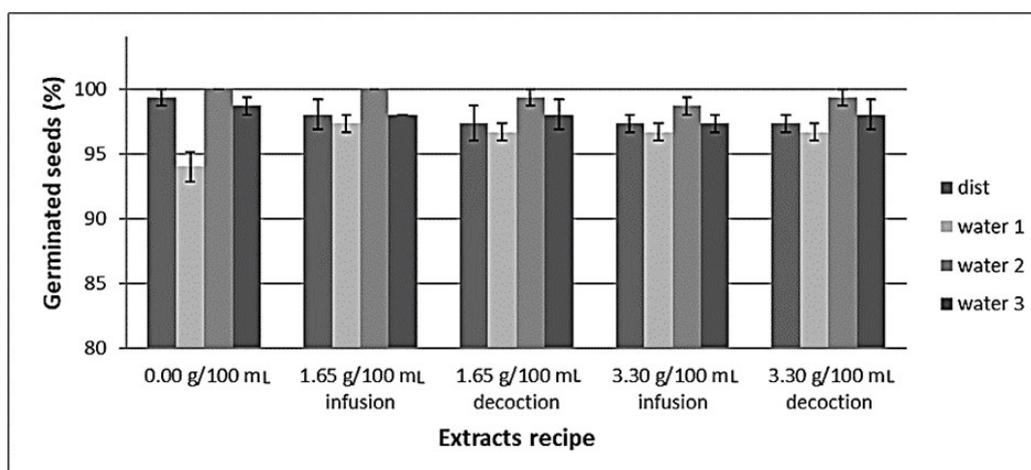


Figure 2. Effect of sage leaf extracts prepared using different waters origin and their concentration on the radish seed germination after 111 h treatment (dist: distilled water; water 1: tap water; water 2: bottled water; water 3: spring water).

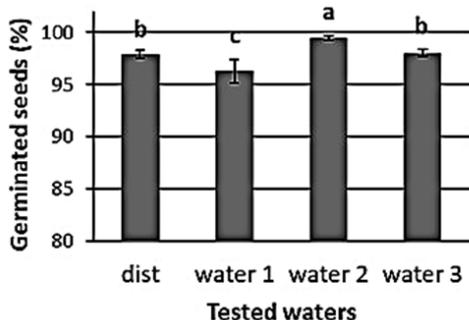


Figure 3. Radish seeds germination after 111 h treatment in distilled water (dist) and waters 1–3 (water 1: tap water; water 2: bottled water; water 3: spring water). Values indicated with different letters differ significantly according to HSD Tukey test

ANOVA:

$$F(3.56) = 13.0, p < 0.0001.$$

differences in values of the S magnitude. In addition, from the presented data a certain dependence of S values (seed germination delay time) on the extracts' preparation way, as well as on their concentration. Differences in values of the S magnitude increase in more concentrated extracts, as can be judged from the calculated p -values: $p = 0.0002$ for extracts with sample of 3.30 g vs $p \cong 0.01$ – 0.02 for extracts with sample of 1.65 g. It should be noted, the pH values of the extracts also change when concentration increases, but have a tendency to converge (Table 1). In the case of thermally unstable bioactive substances, the quality of extracts can be affected by the extraction temperature. With this in mind, extracts were prepared in this study by two ways differing in extraction temperature. Two-way ANOVA was carried out for extracts with sage leaves content 3.30 g/100 mL of water (Figure 5).

Effect of water origin and extract preparation way on S is illustrated in Figure 5(a): ANOVA: $F(3.16) = 48.7, p < 0.0001$ (water origin); $F(1.16) = 24.3, p = 0.0001$ (preparation way); $F(3.16) = 0.5, p = 0.7$ (water origin*preparation way). Figure 5(b) shows the effect of water origin and extract preparation way on the extracts dry rest (DR): ANOVA: $F(3.16) = 3.2, p = 0.0534$ (water origin); $F(1.16) = 17.6, p = 0.0007$ (preparation way); $F(3.16) = 5.3, p = 0.01$ (water origin*preparation way). Effect of water origin and extract preparation way on S/DR value is presented in Figure 5(c):

ANOVA: $F(3.16) = 194.5, p < 0.0001$ (water origin); $F(1.16) = 0.9, p = 0.36426$ (preparation way); $F(3.16) = 0.5, p = 0.7$ (water origin*preparation way). Figure 5(d) illustrates the effect of water origin on S/DR value: ANOVA: $F(3.20) = 118.1, p < 0.0001$.

The tests showed that, in addition to the fact that values of S is significantly affected by the water origin ($p < 0.0001$), there is also a significant effect of the preparation way (decoction or infusion) of the extract ($p = 0.0001$), while there is no interaction effect of these factors ($p = 0.7$).

As can be seen from Figure 5(a), the S values are higher everywhere for the extracts prepared by the "decoction" way than for the extracts prepared by the "infusion" way but an extract preparation process contains subjective points such as the duration of boiling or infusion, the thoroughness of squeezing, etc. To exclude such moments, the content of dry rest in the extracts was taken into account (Figure 5(b)). The data analysis shown a significant effect of the extracts way preparation ($p = 0.0007$), competing with the water origin ($p = 0.0534$) and these factors' interaction ($p = 0.01$) on the dry rest amount. That is, the differences in the extracts dry rest are mainly related to their preparation way. We took into account these differences by normalizing the delay time of seed germination by the amount of the dry rest: S/DR, where DR is the dry rest of extracts, calculated in grams per liter (Figure 5(c)). Based on the data obtained, the S/DR magnitude is significantly affected by the water origin ($p < 0.0001$). The effect of the extracts way preparation and these factors' interaction is insignificant ($p = 0.36426$ and 0.7 , respectively). This means that for all waters, sage leaf extracts prepared in both ways do not differ in quality. Thus, it can also be concluded that for sage, fifteen-minute boiling water extraction is a more productive technology than twenty-four-hour maceration in water at room temperature.

Then, we unified the S/DR data based on the water origin and analyzed it with the larger sample size, similar to data in Figure 3. Figure 5(d) shows the result of this unification. The S/DR magnitude can be considered as an indicator of the biological activity of the extracts in relation to the delay time of seed germination. This indicator allows distinguishes extracts prepared using different

waters in terms of their biocide effect on radish. Note that, within the statistical error, the amount of dry matter in the extracts does not depend on the water origin, and the data for different waters in Figures 5(a) and 5(c), and hence Figure 5(d) correlates to a certain extent.

Taking into account the DR value, the differences in extracts quality become more apparent. The extracts prepared using distilled and spring water showed the best result in terms of its allelopathic properties (Figure 5(d)). In terms of S/DR, the efficiency of distilled water exceeded the efficiency of water 3 by 1.07 times, and for water 1 this excess was 1.36 times. The lowest value was obtained for water 2, whose S/DR is 1.49 times lower than that of distilled water.

3.4. Biological Activity of The Aqueous Plant Extracts

The seed germination test used in this work is one of the most common biological methods for studying allelopathy. Known, the efficiency of

aqueous extraction of biologically active compounds (polyphenols, flavonoids, etc.) from plant materials depends on many factors, including the water minerals content. In most cases, high concentrations of polyphenols and flavonoids are observed in decoctions prepared using waters with low mineralization (deionized or spring water) [32] [42]. Thus, according to Wyrostek and Kowalski, the highest polyphenols and flavonoids content was obtained in decoctions prepared using deionized water [32]. In decoctions with an average mineralization level (bottled water), the biologically active substances content was ~5.8% less. In tap water, the content of polyphenols and flavonoids was ~13% less. The authors attribute this to the large amount of dissolved mineral salts in tap water, which can inhibit the extraction process by reacting with polyphenolic compounds contained in plant materials. It is precisely because of the high mineralization level the tap water is extremely rarely used for the preparation of plant extracts [43] [44]. Ma et al., at studying the catechin content in

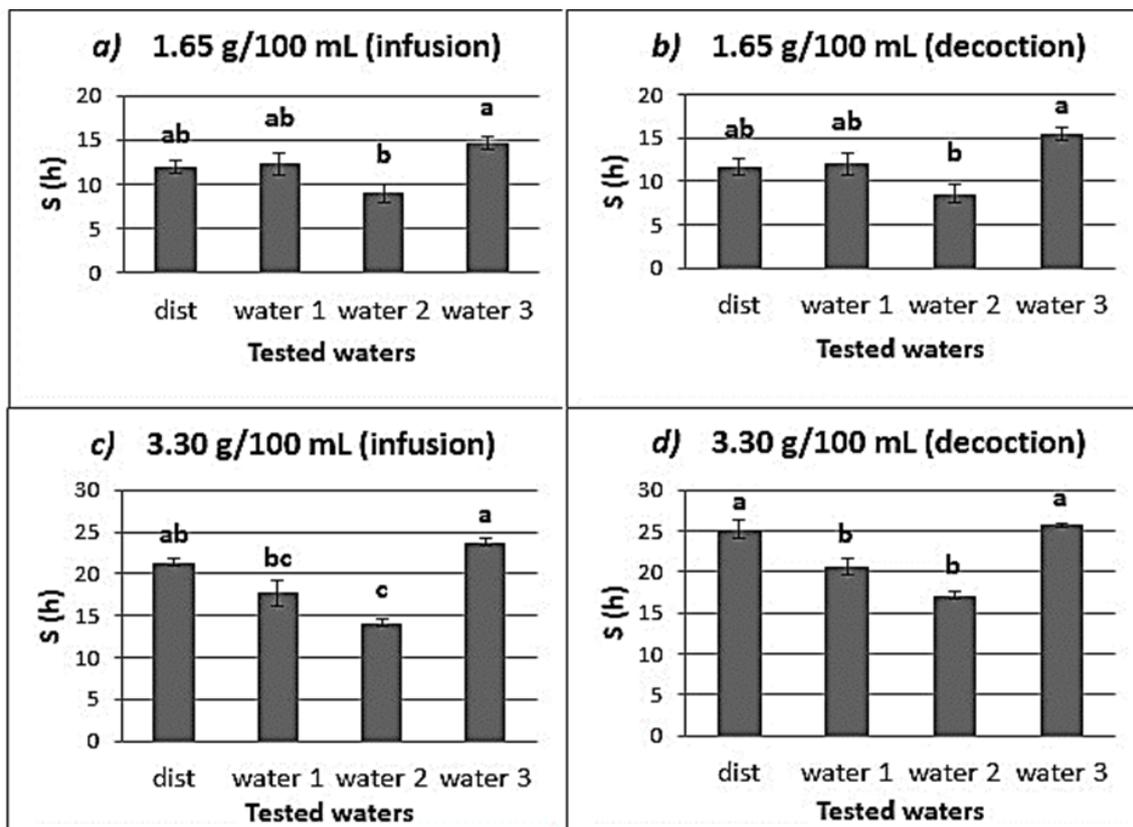


Figure 4. Effect of the extracts concentration prepared with different water on the radish seed germination delay time (S): distilled water; water 1: tap water; water 2: bottled water; water 3: spring water. Values indicated with different letters differ significantly, according to HSD Tukey test ANOVA: a) $F(3.8) = 6.2, p = 0.0176$; b) $F(3.8) = 7.2, p = 0.011$; c) $F(3.8) = 23.7, p = 0.0002$; d) $F(3.8) = 25.6, p = 0.0002$.

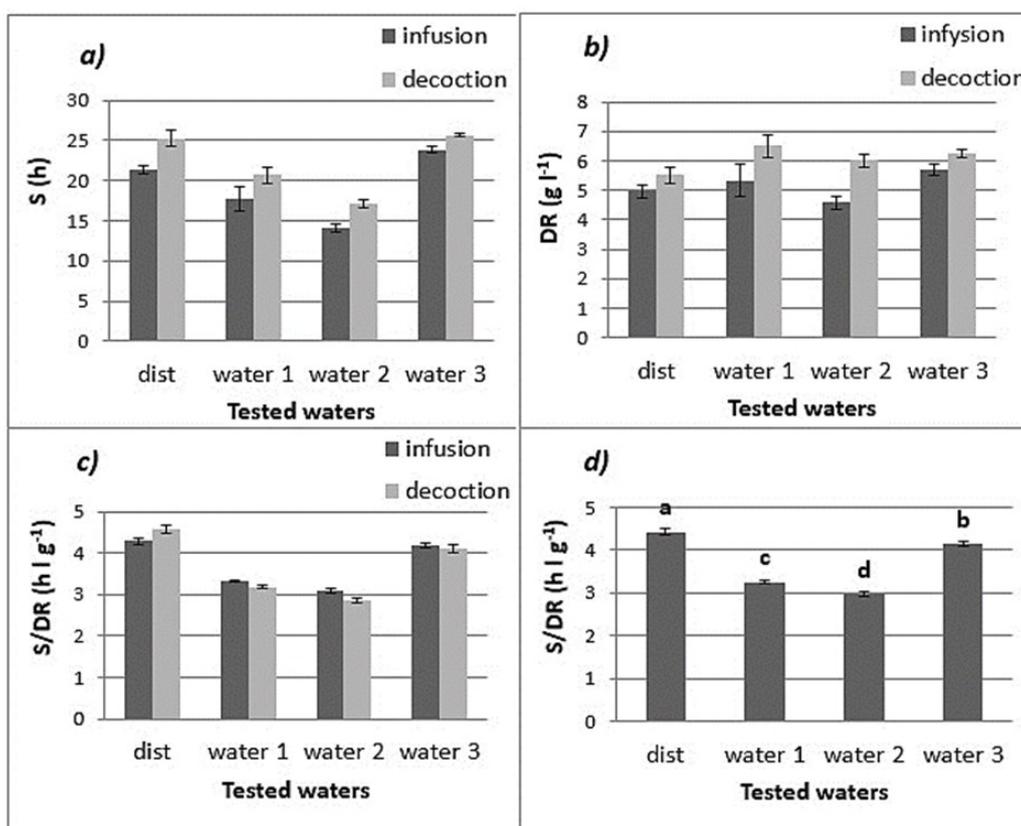


Figure 5. Effect of the sage leaf extracts prepared using different waters origin and the extract preparation way (decoction or infusion) on the radish seed germination. Values indicated with different letters differ significantly, according to HSD Tukey test.

tea extracts obtained using water with different mineralization level (pure water, mountain spring water, mineral water and natural water), showed that water with a high mineral content negatively affects the catechins concentration in the tea infusion [31]. The highest catechins concentration was observed in tea infusions brewed with low-mineralized mountain spring water. In extracts prepared with mineral water, the catechin content was of 3.5-fold lower. In our tests, the significant allelopathic effect on radish seed germination was demonstrated by water 1 (tap water). Sage extracts prepared with spring water had minimal allelopathic effect on radish seed germination, which is consistent with the results of the above studies.

In addition to the solved mineral compounds in the waters, the brewing temperature significantly affects the biologically active substances content in herbal infusions and their properties [32][45]. Moomin et al. reported that metabolites concentration in cold extracts (63%, $p < 0.05$) is higher than in hot ones [46]. In contrast, Findura et al., in a study of the allelopathic properties of

aqueous extracts from 20 plants on the cauliflower seed, not found the significant differences in the influence of the extract preparation methods [47]. In our experiment, boiling water extraction is a more efficient technology than long-term maceration in water at room temperature, as follows from the parameter S at Figure 5(a). The values of parameter S are higher for extracts prepared by the decoction than for extracts prepared by the infusion for all types of water. Thus, the value of parameter S for water 1 is 14.3% higher for the extract prepared by decoction, compared to the extract prepared by infusion. For water 3, the difference in the delay time of seed germination is 7.7%.

Regarding the influence of the extracts' concentration on their allelopathic properties, a number of studies indicate a stimulating effect of extracts at low concentrations and an inhibitory effect at high concentrations [48]-[50]. In our experiment, the stimulating effect of sage extracts on the radish seeds germination in the studied concentration range was not established. But, based on the findings of the above-mentioned works, it

can be assumed that it can appear at a concentration of less than 1.65 g/L. Sage extracts studied in our work at concentrations of 1.65 and 3.30 g/L significantly have affected the delay in the radish germination time compared to distilled water (control). This was in line with the findings of Mozdzen et al. about influence of different nipplewort aqueous extracts concentrations on radish seed germination [51]. It was shown the extracts of nipplewort (*Lapsana communis* L. subsp. *communis*) at concentrations of 3% and 5% inhibited the radish seeds germination compared to the control. An increase in germination was observed for seeds treated with 1% extracts.

In our tests, the S/DR ratio was used to evaluate the effectiveness of sage aqueous extracts as an herbicide against radish. Aqueous extracts of sage prepared using different waters differed in their allelopathic effect on radish seeds from 1.07 (spring water) to 1.49 (tap water) times compared to distilled water as shown the results of our studies. That is, using the same amount of plant material for the extract preparation with herbicidal action, we can achieve fifty percent greater effect with the right choice of water. It should be noted that in this study the choice of radish as the recipient plant and sage as the donor plant, as well as the tested waters was random. The choice of other studies objects, perhaps, might have yielded results with larger differences.

Certainly, each specific case of using aqueous plant extracts requires a separate study of the water's extraction properties. The reason for the water influence on the sage extracts quality may also be related to the plant material composition. Known, the composition of biologically active substances in sage extracts depends on the area where the plant grew and on cultivation technology [39]-[41]. Similarly, the biological properties of water are affected by the dissolved ions of inorganic salts with different composition, which depends on the water origin. This can be argued on the basis of our experiments results on the radish seeds germination presented in Figures 2 and 3. The differences between these results are completely determined by the water origin, as stated above.

4. CONCLUSIONS

The biological properties of sage leaves aqueous extracts were studied in the present work in terms of their dependence on the water origin used for their preparation. Natural waters have different mineral compositions, and these differences affect the allelopathic properties of extracts, which were estimated by the delay time of radish seed germination and by the percentage of seed germination. The allelopathic effect of extracts prepared with tap water was ~1.5-fold higher compared to the effect of extracts based on spring water. The origin of water in the sage extracts significantly influenced the delay time of seed germination and this effect was enhanced for more concentrated extracts. The influence of the extracts preparation methods was also studied. The results showed that extracts preparation way by decoction is a more efficient than infusion for all types of water. Thus, the water origin used for the extracts preparation affects the qualitative composition and mass of the substance extracted from the plant. And, it can be achieved a greater biological effect with a smaller amount of extracted biologically active substance by choosing the right type of water for extraction.

AUTHOR INFORMATION

Corresponding Author

Irina Korotkova — Department of Biotechnology and Chemistry, Poltava State Agrarian University, Poltava-36003 (Ukraine);

 orcid.org/0000-0003-0577-9634

Email: 2irinakorotkova10@gmail.com

Authors

Tamila Romashko — Department of Biotechnology and Chemistry, Poltava State Agrarian University, Poltava-36003 (Ukraine);

 orcid.org/0000-0002-9777-4189

Oleg Khakhel' — Department of Biotechnology and Chemistry, Poltava State Agrarian University, Poltava-36003 (Ukraine);

 orcid.org/0000-0002-6635-0906

Tamila Zvenihorodska — Department of Surgery and Obstetrics, Poltava State Agrarian University, Poltava-36003 (Ukraine);

orcid.org/0000-0002-4186-5700

Tetiana S Yaprnets — Department of Plants, Poltava State Agrarian University, Poltava-36003 (Ukraine);

orcid.org/0000-0002-5844-3520

Viktor Liashenko — Department of Construction and Professional Education, Poltava State Agrarian University, Poltava-36003 (Ukraine);

orcid.org/0000-0003-0177-6209

Author Contributions

Conceptualization, I. K., T. P., O. K.; Methodology, O. K.; Software, V. L.; Validation, I. K., Y. T. S.; Formal Analysis, T. P.; Investigation, T. P., T. Z.; Resources, V. L.; Data Curation, V. L.; Writing – Original Draft Preparation, T. P., Y. T.; Writing – Review & Editing, I. K., O. K.; Visualization, Y. T. S.; Supervision, I. K.; Project Administration, O. K. All authors went through the manuscript and gave their consent for publication.

Conflicts of Interest

The authors declare no conflict of interest.

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