© by PSP Volume 31– No. 02/2022 pages 2250-2258 Fresenius Environmental Bulletin

THE EFFECT OF PLANT EXTRACTS ON SHIKIMIK PATHWAY IN WEEDS *Avena fatua Bromus rigidus* AND *Convolvulus arvensis* IN WHEAT

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ABSTRACT

Decreased wheat crop yields due to the presence of weeds, insufficiently effective herbicides, and the development of weed resistance to high-efficiency herbicides indicated the need to develop alternative ways to control weeds. Therefore, we sought a possible solution in examining the efficiency of different concentrations (1, 5, 10 and 20%) of aqueous extracts of *Ambrosia artemisiifolia* and *Sorghum halepense* in controlling certain weed species for wheat crops: *Avena fatua, Bromus rigidus* and *Convolvulus arvensis*. The efficacy of aqueous extracts through the content of total phenols and four polyphenolic acids (chlorogenic, p-coumaric, ferulic and sikimic) on the level of shikimic acid concentration in treated weeds was investigated. Statistical analysis of the data showed that all tested concentrations of aqueous extracts affect the change in the concentration of sikimic acid, especially the 20% solution of *A. artemisiifolia*. Conducted research also showed that method of monitoring the content of shikimic acid is adequate for determining the herbicidal effect of plant aqueous extracts.

KEYWORDS:
Shikimic acid, plant extracts, weeds, wheat

INTRODUCTION

All plants contain "primary" substances for growth and development (proteins, carbohydrates, chlorophyll and lipids) and "secondary" metabolites (alkaloids, tannins, phenols, etc.) [1] which are not necessary for basic life functions, but play a very important role in the defense mechanism of plants. Particularly important compounds are polyphenolic acids. Their role is twofold, they represent the natural defense mechanism of plants under stress conditions [2] and possess high allelopathic activity [3]. These metabolites can act directly on other plants or through soil [4]. Also, some of these substances may simultaneously have allelopathic and stimulatory effects on surrounding plants [5]. A large number of weeds have been found to exhibit allelopathic activity, which is associated with secondary metabolites, making them more competitive with other plant species [3]. In general, the existence of allelopathy in weeds can be well utilized in pest control in agricultural practice in order to rationalize the use of pesticides in general [6, 7, 8].

Biosynthesis of polyphenolic compounds in plants takes place via two biosynthetic pathways: the shikiminic acid pathway, whose products are mainly phenylpropanoids, and the acetic acid pathway, whose products are simple phenols. Shikimic acid occupies a central place, as an important precursor for the biosynthesis of a large number of other metabolites, the most important of which are amino acids [9, 10, 11]. Precisely because of these characteristics, the presence of sikimic acid can be considered as a measure of plant sensitivity to stress factors (eg herbicides, bioherbicides) [12, 13, 14] or as a metabolite with herbicidal action [15].

The aim of the research was to examine the allelopathic effect of aqueous extracts of weeds Sorghum halepense and *Ambrosia artemisiifolia* on other species, often present in small grain crops: *Avena fatua, Bromus rigidus* and *Convolvulus arvensis*. Especially in the system of organic production, it is important to remove weeds from the budding phase to the appearance of the first knee [16]. These species are difficult to remove from the system of conventional production where herbicides are applied. *A. fatua* is difficult to control because it grows unevenly and there are not enough effective herbicides to remove it. Its impact on yield can range from 2 to 70% [17, 18, 19, 20, 21, 22]. Also, it often happens that plants of this species outgrow wheat plants, which further complicates the good pouring of grain [23].

On the other hand, the species *B. rigidus*, regardless of its abundance, is a strong competitor,

because it develops a good root system (102-305 seedlings/m^2 reduces the yield by 30-50%) [24, 25]. The biggest problem in wheat crops is the species *C. arvensis*: (i) by its number (20-140 seedlings/m^2 reduces the yield by 28-56%), (ii) habitus (crop lodging, difficult harvest), (iii) belongs to the perennial species difficult-to-control weeds [26, 27] and (iv) hosts a number of cereal pests in general [28, 29, 30, 31]. Also, these species have developed resistance to most of the registered herbicidal active substances, which leads to finding an alternative way to control these species.

MATERIALS AND METHODS

Plant materials. Weed seeds were collected in wheat fields in Libya in 2018 and stored at 5 ^{0}C until laboratory tests were performed (Faculty of Futura, 2019). The plants were sown in pots (1 L) filled with substrate for growing seedlings (white peat, Floragard TKS 1). The pots were stored under controlled conditions (phytotron, day/night 12/12, T = 25 ^{0}C) and watered as needed. After germination, the plants were thinned to 3 per pot. The application of aqueous extracts of *A. artemisiifolia* (AA) and *S. halepense* (SH) (1, 5, 10 and 20% solution) and herbicides (am bentazone, piroxulam, glyphosate) was done with a hand sprayer with a volume of 500 ml in the phase when wheat and grass species were 10-15 cm tall, and *C. arvensis* in the stage of 4-6 leaves. Sampling sheets for analysis were done before treatment, 3 and 6 days after treatment (DAT).

Plant extracts. Plants for making aqueous extracts (AA and SH) were obtained from seeds under controlled conditions. The seeds were sown in pots with a volume of 2 L filled with substrate for growing seedlings, the pots were stored in constant conditions (above), watered as needed until the lush mass increased. After harvest, the plant material (aboveground part) was ground in liquid nitrogen and a 20% (w/v) aqueous solution was prepared. Extraction of bioactive compounds was performed in an ultrasonic bath (A-sonic PRO 60, frequency 40kHz) for one hour (30 + 30 min with a break of 15 min), after which filtration was performed (Whatman). From the initial solution, working solutions with concentrations of 10, 5 and 1% (w/v) were made by adequate dilution. All solutions were made immediately before application, and distilled water was used for extraction and dilution.

Extraction of polyphenols from plant material AA and SH. Extraction of polyphenolic compounds from plant material was performed as follows: 3 g of plant material was weighed on an analytical balance with an accuracy of 0.00001 g for each plant species, crushed in liquid nitrogen, and extracted with 10 ml of 70% methanol (aqueous solution). The extraction was performed in an ultrasonic bath for 1 h, and then the samples were additionally left overnight at room temperature. After 24 h, the samples were filtered (0.45 µm diameter PTFE filter) and stored in a refrigerator at 4 ^{0}C until analysis.

Determination of total phenols content. From prepared extracts, content of total phenols was determined spectrophotometrically, using Folin-Cioucalteu reagent and gallic acid standard according to the previously optimized method [32].

Quantification of individual polyphenolic acids. Analysis of polyphenolic acid content was performed on a liquid chromatograph (Shimadzu Nexera XR) according to the previously optimized method [32]. Separation of polyphenolic acids was performed on a column Zorbax SB C18 4.6 × 250 mm, pore diameter 5 µm, thermostated at 25 ^{0}C. The flow of the mobile phase was 1 ml/min, 10 µl of the sample was injected, and the reading was performed at a wavelength of 325 nm. Quantification was performed by the external standard method. A solution of ferric, p-coumaric and chlorogenic acid at a concentration of 1 mg/ml in 70% MeOH was prepared, from which further dilutions were made. Calibration lines in the concentration range of 10 to 150 µg/ml were constructed for each acid tested. Extraction was performed for each species in three replications, and the results were presented as the mean.

Extraction and quantification of shikimic acid content. 1.5 g of plant material (*A. fatua, B. rigidus, C. arvensis, S. halepense, A. artemisiifolia*) were weighed, ground in liquid nitrogen, and extracted with 10 ml of 0.1 M HCl each. The extracts were shaken on a shaker for 24 hours in the dark. The material was then filtered (PTFE filter, 0.45 µm) and adjusted to 3-3.5 pH with 1M and 0.01M NaOH solutions. Measurement of shikimic acid concentration was performed on a liquid chromatograph (Shimadzu Nexera XR) according to the method [33]. Analyzes were performed on a Polaris 5 NH_2 column, 4.6 × 250 mm, pore diameter 5 µm, thermostated at 25 ^{0}C. The flow of the mobile phase was 1 ml/min, 10 µl of extracts were injected. The reading was performed at a wavelength of 215 nm, and the elution time of shikimic acid was about 7 minutes. Quantification was performed by the external standard method. A solution of shikimic acid at a concentration of 1 mg/ml was prepared, from which further dilutions were made. The calibration curve was constructed in the concentration range from 5 to 100 µg/ml. Extraction was performed for each species in three replications, and the results were presented as the mean.

All obtained results were processed by analysis of variance (Duncan test, ANOVA, StatSoft 8).

RESULTS AND DISCUSION

The Shikimic pathway links primary and secondary metabolism in plants [1] and provides the synthesis of amino acids necessary for the construction of proteins in plants, fungi, and bacteria. Under stress conditions, plants activate one of their defense mechanisms through this biochemical process [1, 34]. During this process, various enzymes are active through which the stress factor can disrupt the process itself. Most often, blocking the enzyme 5-enolpyruvate-shikimat-3-phosphate synthetase (EPSPS) leads to the accumulation of sikimic acid, which results in a lack of amino acid (protein) synthesis, ie plant decay [12, 13].

It is known that some herbicides bind to the enzyme EPSPS [14]. The hypothesis of the performed experiments was to examine the herbicidal activity of *A. artemisiifolia* and *S. halepense* extracts on the activity of EPSP enzymes. Allelochemicals in plants can act on more than one cellular process: photosynthesis, cell differentiation and division, enzyme function, and others [35, 36, 37]. Based on the effect of herbicidal active substances on the concentration of sikimic acid, we tried to define the mechanism of action of aqueous extracts of both species. In the experiments we did not analyze the content of polyphenolic acids in treated plants (only in AA and SH extracts), except sikimic acid, but some studies have confirmed that in plants *A. fatua* and wheat, their excretion is through the root system and negatively affects seedlings of the same species in the environment [38, 39, 40]. This mode of action is insufficiently elucidated [41].

Analysis of the content of free polyphenolic acids in aqueous extracts of AA (*A. artemisiifolia*) and SH (*S. halepense*) showed that there are more of them in the extracts of SH (Table 1). The percentage content of chlorogenic, coumarin, p-ferulic and sikimin in AA extracts is 51.92% and in SH extracts 10.87%. Chlorogenic acid (44.01%) was the most abundant in AA extracts, while p-coumaric acid (2.99%), ferulic acid (4.35%) and sikimic acid (0.58%) were present in a significantly lower concentration. In extracts of the plant species *S. halepense* (SH) the content of chlorogenic and ferulic acid was balanced (4.84 and 4.99%, respectively), sikimic acid is present in a lower concentration (1.04%), while coumaric acid was not detected (Table 1).

Based on the analysis of plant material (AA and SH) for making extracts, it is concluded that their action as herbicides can be related to the content of total phenols (extract AA 1082.4 μg/g fresh weight, SH 1373.9 μg/g fresh weight), and the amount free polyphenolic acids: chlorogenic, p-coumaric, ferulic and sikimic (Table 1). Our research is consistent with the conclusions reached by Bruckner [42] and Wang and Zhu [43]. The authors explain that the allelopathic activity of *A. artemisiifolia* is based on phenolic compounds and terpenes.

Also, they state that aqueous extracts of nadyemnog part affect the germination and primary growth of wheat and mayze. Similar studies conducted for species of the genus *Sorghum* indicate that allelopathic activity is associated with isolated polyphenolic acids (ferulic, p-coumaric, vanillin, etc.) [44]. The main difference in our research was that SH aqueous extracts contain a higher amount of total phenols compared to AA aqueous extracts (Table 1). However, although the content of total phenols in SH extracts was higher, the herbicidal activity of the solution could not be related to the determined amounts of individual acids (chlorogenic 4.84%, ferric 4.99%, p-coumarin 0% and sikimic 1.04%). In contrast to SH solutions, in AA solutions the herbicidal activity is associated with the chlorogenic acid content, 44.01% of the total total phenols content (1082.4 μg/g fresh weight, Table 1).

Treatment of weed plants with different concentrations of aqueous AA extract in *A. fatua* species led to an increase in the concentration of sikimic acid 6 DAT compared to the control (1.57x, 1.55x, 1.18x and 1.21x; Table 2; Figure 1a). Also, all tested concentrations of SH extract affected the accumulation of sikimic acid 6 DAT: 1.57x, 1.29x, 1.27x and 1x (Table 2; Figure 1b) in the treated plants. No dependence of the change in the concentration of shikimic acid with the change in the concentration of the solution after the application of both extracts was observed.

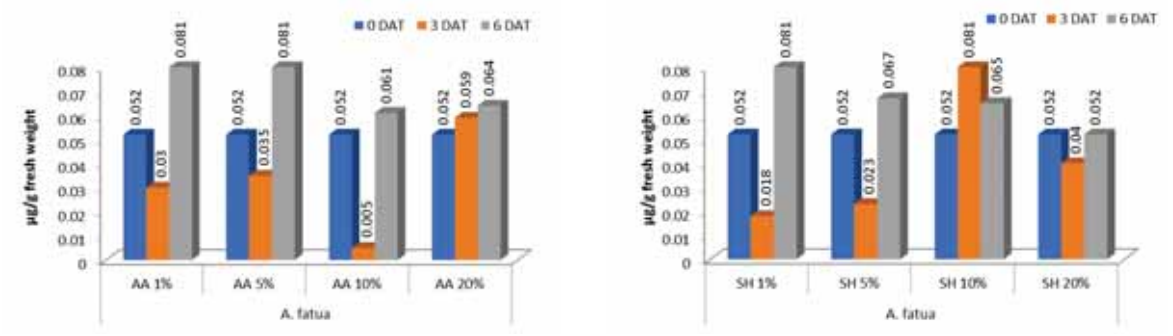
TABLE 1
Concentration of polyphenolic acids in plant material *A. artemisiifolia* and *S. halepense*

	chlorogen	p-coumaric	ferulic	shikimic	total phenols
	μg/g fresh weight				
AA	371.09	31.21	40.89	5.74	1100.6
	595.38	36.01	56.65	7.03	1074.6
	462.72	29.99	43.75	5.97	1072.0
average	**476.39**	**32.40**	**47.11**	**6.25**	**1082.4**
SH	67.03	0	69.63	15.38	1708.0
	67.78	0	69.94	13.45	1315.1
	64.55	0	66.25	13.91	1098.8
average	**66.45**	**0**	**68.61**	**14.25**	**1373.9**

TABLE 2
Average values of shikimic acid concentration (µg/g fresh weight) after application of different concentrations of aqueous extracts

	C	3 DAT	6 DAT	C	3 DAT	6 DAT	C	3 DAT	6 DAT
AA		*A. fatua*			*B. rigidus*			*C. arvensis*	
1%	0.051	0.030	0.080**	0.037	0.058	0.052**	0.051	0.095	0.078**
5%		0.035	0.079**		0.064	**0.110****		**0.122**	0.006**↓
10%		0.005	0.060**		0.031	0.050**		0.066	0.005**↓
20%		0.059	0.062**		0.038	0.043**		0.069	0.081**
SH		*A. fatua*			*B. rigidus*			*C. arvensis*	
1%	0.051	0.018	0.080**	0.037	0.034	0.055**	0.051	0.057	0.066**
5%		0.023	0.066**		0.020	**0.104****		0.066	0.051ns
10%		0.081	0.065**		0.006	0.050**		0.055	0.053ns
20%		0.040	0.051ns		0.059	0.031**↓		0.076	0.052ns

C-control, AA-*A. artemisiifolia*, SH-*S. halepense*, DAT day after treatment, p<0.05*, p<0.01**; ↓ low level then in control; ns-no significant



a) b)

FIGURE 1
Concentration of *shikimic acid* in *A. fatua* leaves after application of extract a) *AA-A. artemisiifolia* and b) *SH-S. halepense*

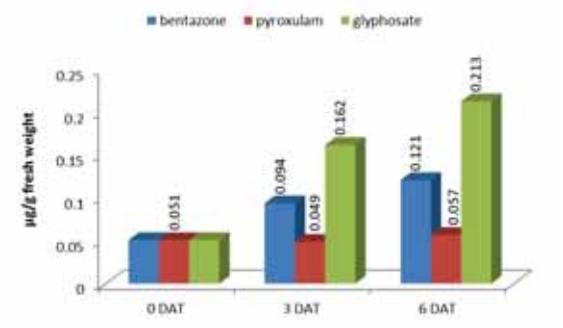


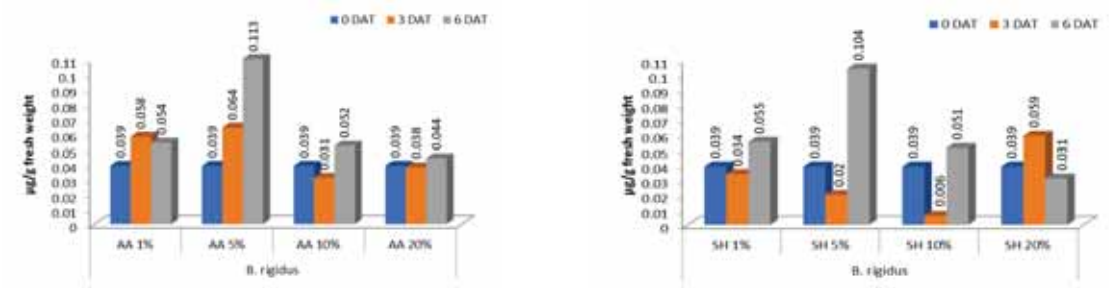
FIGURE 2
Sikimic acid concentration in leaves *A. fatua* after application a.m. herbicide

Statistical analysis of the obtained results showed that all applied concentrations of both extracts led to a statistically significant increase in the concentration of sikimic acid 6 DAT (except for the use of 20% solution of *S. halepense*, Table 2).

Comparison of the action of herbicides and different concentrations of aqueous extracts AA and SH showed that in species *A. fatua* herbicidal a.m. bentazone (magnification 2.33x) and glyphosate (magnification 7.75x) caused a higher increase in sikimic acid concentration than AA and SH solutions, without a.m. pyroxulam caused changes similar to the action of plant extracts (magnification 1.10x) (Figure 2).

In *B. rigidus*, the application of all tested concentrations of both extracts 6 DAT leads to an increase in the concentration of shikiminic acid in relation to the initial state: 1) AA solutions: 1.40x (1% solution), 2.97x (5% solution), 1.35x % solution) and 1.16x (20% solution) (Table 2, Figure 3a) and 2) SH solutions: 1.49x (1% solution), 2.81x (5% solution), 1.35x (10% solution) and 1.19 x (20% solution, less than the control) (Table 2, Figure 3b), which was confirmed by statistical analysis (except for 20% solution SH, Table 2).

Comparison of the action of herbicidal a.m. and different concentrations of aqueous extracts AA and SH in *B. rigidus* showed the following: a.m. glyphosate influenced the appearance of the highest sensitivity of the treated plants, ie there was a significant increase in the concentration of 7.28x sikimic acid (Figure 4). Other tested herbicidal a.m. affected the increase in the concentration of sikimic acid similar to the level of action of the applied aqueous extracts AA and SH (a.m. bentazone magnification 1.91x; a.m. pyroxulam magnification 1.15x) (Figure 3a, b, 4).



a) b)

FIGURE 3
Concentration of shikimic acid in *B. rigidus* leaves after application of extract
a) *AA-A. artemisiifolia* and b) *SH-S. halepense*

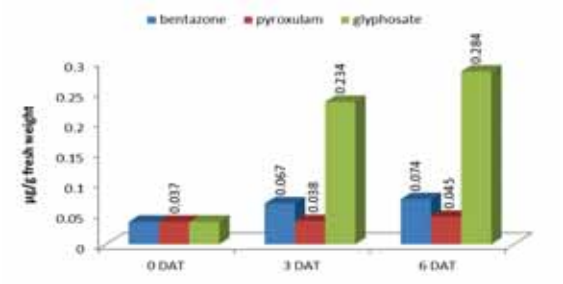


FIGURE 4
Sikimic acid concentration in leaves *B. rigidus* after application of a.m. herbicide

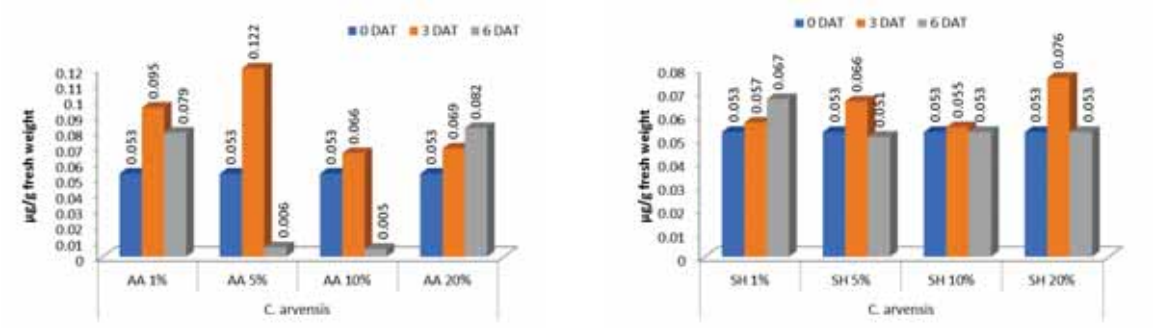
Measured concentration of sikimic acid in *C. arvensis* after application of different concentrations of the extract was: 1) AA: 1.53x increased (1% solution), 8.50x reduced (5% solution), 10.2x reduced (10% solution) and 1.56x increased (20% solution) and 2) SH: 1.29x increased (1% solution) and solutions 5, 10 and 20% did not affect statistically significant changes in sikimic acid concentration (Table 2, Figure 5a, b).

Changes in sikimic acid concentration after application of herbicidal a.m. in *C. arvensis* were much more pronounced (Figure 6) compared to changes caused by the use of aqueous extracts, especially SH (5, 10 and 20%, 6 DAT, Figure 5a, b).

The determined increase in the concentration of sikimic acid in the treated plants shows that the AA and SH solutions have herbicidal activity. Which is also linked to the EPSP enzyme. However, dilute solutions (concentrations of 1, 5 and 10%) are not sufficient for complete weed control.

Measured lower/higher values of sikimic acid 3 DAT in treated weeds in relation to the control show that the plants experienced stress and are trying to overcome this condition (Figure 1a, b; 3a, b; 5a, b). Decreased concentrations may be associated with a slowdown in metabolism, as one way to overcome the current state [45]. Also, plants can increase the amount of certain enzymes and thus neutralize the presence of extracts, herbicides in general [46].

There are studies that show that aqueous extracts can also have a stimulating effect on treated plants [47], which may explain the effect of lower concentrations of the tested AA and SH extracts. The increase in the concentration of 6 DAT (Figure 1a, b; 3a, b) in the performed experiments shows that the plants (except *C. arvensis*) failed to reduce the stress effect caused by the application of the tested extracts (AA and SH). The observed different effect of lower concentrations (1, 5 and 10%) and the lack of correlation between the increase in the amount of application and the measured concentration of sikimic acid can be related to the stimulating/herbicidal action of aqueous extracts, but not to blocking the EPSP enzyme. Some researchers point out that the conclusions should be based on the fact that the concentration of shikimic acid depends on the stage of plant development at the time of evaluation [48] and that it is lost from the tissue over time [49]. In contrast, Sing and Shaner [50] point out that the age of plants does not affect the sensitivity of the method of measuring the concentration of sikimic acid in plants. Also, there are studies that indicate that shikimic acid itself (as a metabolite) may be an allelochemical [15]. In their experiments, the authors showed the inhibitory effect of shikiminic acid (extract from *Illicium verum* Hook.) On the growth of seedlings of *Trifolium pratense, T. repens*, *Medicago sativa* and *Lotus corniculatus*. In our experiments, we did not correlate the effect of the extract based on the concentration of sikimic acid (average content in AA tissue 6.25 and SH 14.25 µg/g fresh weight, Table 1) but with the concentration of chlorogenic acid: in AA tissue average 476.39 µg/g of fresh mass in SH tissue with a concentration of chlorogenic 64.45 and ferric 68.61 µg/g of fresh mass (Table 1). Similar to our results, this is confirmed by the results of Taiz and Zeiger [51]. These authors state that the effect of the examined extracts of *A. artemisiifolia* and *S. halepense* may be related to the synthesis of some phenols in the shikimic acid pathway. This is confirmed by Waniska et al. [52] by measuring a significant concentration of total free polyphenols in the leaves of Sorghum bicolor plants, respectively 63% (concentration in leaves of the total concentration of polyphenols). Once stressed, a plant can produce a certain amount of allelochemicals that can have a negative effect on individuals of the same species [53].



a) b)

FIGURE 5
Concentration of shikimic acid in *C. arvensis* leaves after application of extract a) *AA-A. artemisiifolia* and b) *SH-S. halepense*

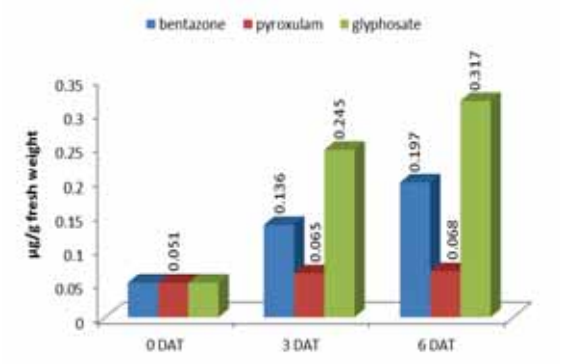


FIGURE 6
Sikimic acid concentration in leaves *C. arvensis* after application of a.m. herbicide

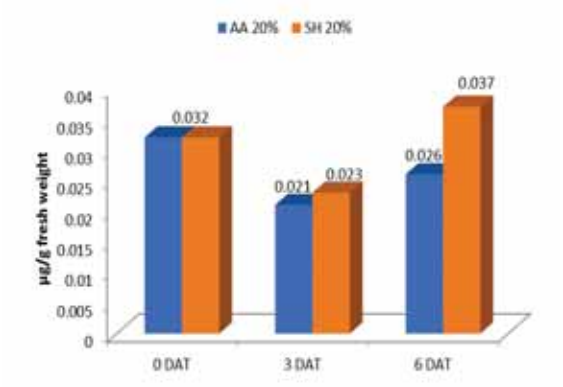


FIGURE 7
Concentration of shikimic acid in wheat leaves after application of 20% of AA-*A. artemisiifolia* and SH-*S. halepense*

The effect of the highest tested concentration of AA and SH solutions on the test plant (wheat) is shown in Figure 7. The analysis showed that the highest tested concentrations 3 and 6 DAT did not cause changes in concentration (except for a slight increase after application of 20% solution of *S. halepense*, 1.1x).

The analysis of the effect of the highest tested concentrations of AA and SH solutions on wheat plants also confirmed the unjustified use of SH solution. A solution of 20% SH led to the accumulation of sikimic acid in wheat plants (71.87%), and a solution of AA to a decrease in content compared to control plants (65.60%), which justifies its use as a solution with herbicidal action similar to a.m. bentazone (Figure 7). In general, research says that wheat's resistance to herbicides is achieved through metabolic processes, uptake and transport, and that it recovers 100% after 24 hours [54], which is related to the action of AA solution.

Graphs 2, 4 and 6 clearly show that the effect of a.m. glyphosate led to a very significant increase in the concentration of sikimic acid in the treated weeds and that none of the concentrations of the tested extracts had a similar effect, which confirms that the extracts are not EPSP inhibitors. Comparison of the highest tested amount of both extracts showed that 20% AA leads to a statistically significant accumulation of sikimic acid 6 DAT in all treated species as I am. bentazone. In contrast, 20% SH solution reduced or maintained sikimic acid levels as in control, which does not link it to the mechanism of action of some herbicides (photosynthesis inhibitor, acetolactate synthetase inhibitor, EPSP inhibitor). The herbicidal solution a.m. behaved similarly to the action of 20% SH solution piroxulam. Due to the stated weak effect of SH solution, we conclude that it is not acceptable as an alternative bioherbicide in weed control

CONCLUSIONS

Based on the performed analyzes, it is concluded that weed species are more sensitive to the action of *A. artemisiifolia* extract. The measured values and their statistical analysis confirm the sensitivity of the method of measuring the concentration of shikimic acid to determine the herbicidal effect of aqueous extracts. Also, comparing the obtained results with other methods on the same weed species (SPAD reading, extraction and fluorescence of chlorophyll) confirms the potential herbicidal activity of the tested extracts (presented in Zawia et l. [55]).

ACKNOWLEDGEMENTS

DP, AA and SDJ would like to acknowledge the financial support of the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 451-03- 68/2020-14/200010).

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Received: **02.09.2021**
Accepted: **07.11.2021**

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