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Allelopathic effects and insecticidal activity of aqueous extracts of *Satureja montana* L.

JOVANA ŠUĆUR[1]*, ALEKSANDRA POPOVIĆ[1], MILOŠ PETROVIĆ[1], GORAN ANAČKOV[2], VOJISLAVA BURSIĆ[1], BILJANA KIPROVSKI[1] and DEJAN PRVULOVIĆ[1]

[1]*Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia and* [2]*Faculty of Science, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia*

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Abstract: Extensive use of synthetic insecticides, herbicides and other pesticides has negative effects on the environment and on human and animal health. Therefore, scientists are turning towards natural pesticides, such as active components of plant extracts. The effect of two concentrations (0.1 and 0.2 %) of a *Satureja montana* L. aqueous extract on the lipid peroxidation process, as well as on the activity of the antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPX), pyrogallol peroxidase (PPX) and catalase (CAT) in the leaves and roots of pepper and black nightshade seedlings were examined 24, 72 and 120 h after treatment. The results showed that the higher concentration of *S. montana* aqueous extract induced lipid peroxidation in black nightshade roots. Furthermore, significant increases of pyrogallol and guaiacol peroxidase were detected in black nightshade leaves treated with 0.2 % of the *S. montana* aqueous extract. The second aim was to evaluate effectiveness of the aqueous extract as a contact toxicant against whitefly. It was observed that the 0.2% aqueous extract exhibited a toxic effect with 68.33 % mortality after 96 h.

Keywords: allelochemicals; antioxidants; biopesticides.

INTRODUCTION

The synthetic agrochemicals, such as herbicides, may cause imbalance of soil microorganisms and nutrient deficiency, resulting in decreases in crop productivity. The application of allelochemicals into natural and agricultural practice may reduce the use of herbicides, insecticides, and other pesticides, thereby reducing environmental pollution and diminishing auto-toxicity hazards. Allelochemicals are secondary metabolites of plants and may be present in all plant organs, including leaves, flowers, fruits, roots, rhizomes, stems and seeds.[1,2]

* Corresponding author. E-mail: jovanasucur@yahoo.com
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Allelochemicals extracted from the roots or shoots of plants were shown to directly inhibit or stimulate growth and development of other plants.[3] Some plant extracts imparted insecticidal effects on adult insects by killing them or inducing complete inhibition of the feeding activity of the insect pests.[4]

One of the effects of allelochemicals on target plants, and one of the important mechanism by which plants are damaged, is the excess production of reactive oxygen species (ROS), such as superoxide anions ($O_2^{-}\cdot$), and the more reactive hydroxyl ($^{\bullet}OH$) or hydroperoxyl ($HO_2^{\bullet}$) radicals.[5,6] These plant–plant allelopathic interactions generates a cascade of signaling events, including Ca^{2+} influx and proton efflux that activates the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex (generating $O_2^{-\bullet}$), and pH-sensible cell wall peroxidases (producing H_2O_2), which initiate an oxidative burst.[7] Enhancement of ROS production during an oxidative burst is one of the earliest reactions elicited in response to various abiotic and biotic stimuli. These molecules are very toxic to cells and their excessive production is accompanied by the activation of the cellular antioxidant system.[6,8,9] Thus, H_2O_2 can act as an intercellular messenger to induce some enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT), etc.[10] These enzymes play important roles in protecting a cell against the potentially deleterious effects of reactive oxygen species.[11] Within a cell, the superoxide dismutases (SODs) constitute the first line of defense against ROS.[12] Superoxide dismutases (SODs) are metal-containing enzymes that catalyze the dismutation of superoxide free radical anions, converting them to H_2O_2.[13–15] Plants contain several types of enzymes that are able to metabolize hydrogen peroxide. These include catalases and peroxidases. Catalases are highly expressed enzymes, particularly in certain plant cell types, and are thus an integral part of the plant oxidative system.[16] Catalases are peroxisomal enzymes that, in contrast to peroxidases, do not require a reducing substrate for their activity.[5] Catalases are also distinguished from many other peroxide-metabolizing enzymes by their high specificity for H_2O_2, but weak activity against organic peroxides.[16] The cellular level of H_2O_2 could be sufficiently toxic to inhibit the activity of the enzymes, leaving the plant vulnerable to oxidative damage.[10] Some allelochemicals rapidly depolarize the cell membrane, increasing the permeability of the membrane, inducing lipid peroxidation and causing a generalized cellular disruption that ultimately leads to cell death.[17–19]

Satureja montana L., commonly called winter or mountain savory, belongs to the Lamiaceae family and is native to the Mediterranean regions.[20–22] This aromatic herb, found in nature and also cultivated, is a well known medicinal plant that contains various biologically active constituents, such as, essential oil, triterpenes and flavonoids.[22,23] It has been used in traditional medicine for various diseases, such as: asthma and peptic ulcer, as a diarrheic, antipyretic, anti-inflammatory, antibacterial and antiviral medicine and for its insecticidal acti-

vities.[21] An aqueous extract of winter savory had a high antioxidant capacity.[24] Furthermore, aqueous extracts of *Satureja* species had inhibitory effects on the growth of the root, stem, leaf, shoot, germination rate and germination percentage of seeded weeds, *Chenopodium album* and *Portulaca oleracea*.[25]

Due to an increase in the number of herbicide-resistant weeds and environmental concerns in the use of synthetic herbicides, there have been considerable efforts in designing alternative weed management strategies. Extensive use of synthetic insecticides usually has negative effects on the environment and on human and animal health and resistance develops among insects. Therefore, scientists are turning towards natural insect suppressants.[4]

The aim of this study was to examine the effects of aqueous extract of *S. montana* L. on pepper and black nightshade antioxidant activity and to explore the potential of this species in weed control. The second aim was to evaluate the effectiveness of *S. montana* aqueous extract as a contact toxicant against greenhouse whitefly.

EXPERIMENTAL

Plant material and preparation of the aqueous extract

The wild, aromatic plant, *S. montana* was collected at localities near the Adriatic coast in Montenegro, in June, 2012. Voucher specimens of the collected plant were confirmed and deposited at the Herbarium of The Department of Biology and Ecology, Faculty of Science, University of Novi Sad.

The air-dried plant material was ground into powder. The powdery material (10 g) was extracted with 100 mL distilled water. After 24 h, the extract was filtered through filter paper and kept at 4 °C until application.

Determination of total phenolic and flavonoid contents in S. montana *aqueous extract*

The total phenolic content of *S. montana* aqueous extract was determined according to the Folin–Ciocalteu method.[26] Extract (0.02 mL) was mixed with 3.36 mL of deionized water, 0.4 mL of 20 % sodium carbonate and 0.2 mL of 33 % Folin–Ciocalteu reagent. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 720 nm. The data are expressed as mg gallic acid equivalents g^{-1} dry weight (mg GA equivalents g^{-1} d.w.).

The total flavonoids were estimated according to the method described by Markham.[27] Extract (0.4 mL) was mixed with 1 mL of deionized water and 2.5 mL of 2 % aluminum chloride hexahydrate solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. The data are expressed as mg rutin equivalents g^{-1} dry weight (mg rutin equivalents g^{-1} d.w.).

Determination of phenolic compounds in S. montana *aqueous extract*

Chemicals and apparatus. All solvents used were of chromatography grade and were obtained from J. T. Baker (Deventer, The Netherlands). Ferulic acid (99.0 %), *trans*-cinnamic acid (99.0 %), gallic acid (99.9 %), caffeic acid (98.0 %), 2-hydroxycinamic acid (97.0 %), *p*-coumaric acid (98.0 %), chlorogenic acid (95.0 %), quercetin (98.0 %) and kaempferol (97.0 %), all Sigma–Aldrich, were used as analytical standards. The stock standard solutions were prepared by dissolving the required analytical standard in methanol, while the working

solution, *i.e.*, the mixture of the studied phenol compounds, was obtained by mixing and diluting the stock standards with mobile phase, resulting in a final mass concentration of 100 μg mL^{-1}. The composite mixtures of all phenol compounds at appropriate concentrations were used to spike samples in the data validation settings. Acetic acid was of *p.a.* grade (Carl Roth).

HPLC analysis. The chromatographic separation for phenolic compounds was achieved using an Agilent 1100 (Agilent Technologies, USA) HPLC system with a binary pump and diode array detector - DAD. The phenolic acids were separated on a ZORBAX SB-Aq (5 μm particle size: 4.6 mm×250 mm, Agilent) column. The aqueous extract was filtered through 0.45-μm syringe filters and directly injected through a 30 μL fixed loop onto the column.

The mobile phase was acetonitrile with 2.0 % acetic acid (solvent A) and Milli-Q water with 2.0 % acetic acid (solvent B) in gradient mode, at a flow rate of 1.0 mL min^{-1}. The gradient was as follows: 92 % A at 0 min, 80 % A at 18 min, 60 % A at 25 min, 55 % A at 30 min, 35 % A at 40 min and 20 % A at 42 min. Stop time was 2.5 min.

Validation parameters. The repeatability of the method was determined by analyzing a sample of the same mass concentration level (10.0 μg mL^{-1}) in six replicates and shown through the relative standard deviation (*RSD*). The detection limit (*LOD*) was defined as the amount of phenolic compounds that produces a signal three times the noise signal. The limit of quantification (*LOQ*) is the amount of phenolic compounds that produces a signal ten times the noise signal. The *LOD*s were determined by adding 100 μL of the standard mixture of the phenol compounds to a concentration of 1.0 μg mL^{-1} into 0.5 g of the sample in six replicates and the *LOD*s were calculated.

Seedling growth

The pepper (*Capsicum annuum* L.) cultivars Anita and black nightshade (*Solanum nigrum* L.) seeds were grown in a controlled climate chamber at 28 °C, 60 % relative humidity, a photoperiod of 18 h, and a light intensity of 175 μmol m^{-2} s^{-1} in plastic pots containing sterile sand. After 30 days, the seedlings were transplanted into plastic pots containing 700 mL of Hoagland solution prepared according to Hoagland and Arnon,[28] and 7 and 14 mL of *S. montana* aqueous extract, while the pots of the control contained the same volume of Hoagland solution. Seedlings were harvested for further biochemical analyzes 24, 72 and 120 h after the treatments.

Enzyme extraction

Fresh leaves and roots (2 g each) were homogenized in 10 mL of phosphate buffer (0.1 M, pH 7.0). The homogenates were centrifuged for 20 min at 10,000×*g* and filtered. The supernatants were used to test enzyme activity and to determine intensity of lipid peroxidation.

Membrane lipid peroxidation

Lipid peroxidation was measured at 532 nm using the thiobarbituric acid (TBA) test. The enzyme extract (0.5 mL) was incubated with 2 mL of 20 % trichloroacetic acid (TCA) containing 0.5 % thiobarbituric acid for 40 min at 95 °C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000×*g* for 15 min. The total amount of TBA-reactive substances is given as nmol malondialdehyde (MDA) equivalents mg^{-1} protein.[29]

Assay of catalase activity

The catalase (CAT) (EC 1.11.1.6) activity was determined according to Sathya and Bjorn.[30] The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm. The

enzyme extract (0.02 mL extract of leaves or 0.1 mL extract of roots, separately) was added to the assay mixture containing 1 mL for leaves and 1.5 mL for roots of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The activity of the enzyme is expressed as U per 1 g of protein (U g^{-1} protein).

Assay of superoxide dismutase activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed according to a slightly modified method of Mandal *et al*.[29] by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.02 mL of the enzyme extract. It was kept under a fluorescent lamp for 30 min, and then the absorbance was read at 560 nm. One unit of the SOD activity is defined as the amount of enzyme required to inhibit the reduction of NBT by 50 %. The activity of the enzyme is expressed as U per 1 mg of protein (U mg^{-1} protein).

Assay of peroxidase activity

Peroxidase (EC 1.11.1.7) activity was measured using guaiacol (guaiacol peroxidase; GPX) and pyrogallol (pyrogallol peroxidase; PPX) as substrates according to Morkunas and Gmerek.[31] The peroxidase activity (GPX and PPX) is expressed as U per 1 mg of protein (U mg^{-1} protein).

Pyrogallol peroxidase activity. This method is based on the measurement of the content of purpurogallin – a product of pyrogallol oxidation. The enzyme extract (0.02 mL) was added to the assay mixture containing 3 mL of 180 mM pyrogallol and 0.02 mL of 2 mM H_2O_2. The absorbance was recorded at 430 nm.

Guaiacol peroxidase activity. This method consists of an assay of tetraguaiacol – a colored product of guaiacol oxidation in the investigated sample. The enzyme extract (0.04 mL) was added to the assay mixture containing 3 mL of 20 μM guaiacol and 0.02 mL of 3 mM H_2O_2. The absorbance was recorded at 436 nm.

Insects

The experiment on the adult of whitefly, *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae), collected in a greenhouse, was performed at the Faculty of Agriculture, University of Novi Sad.

Toxicity test

The bioassays were realized using groups of 20 adult insects *T. vaporariorum*, kept in transparent laboratory dishes (25 cm×12 cm), fed on the pepper nursery plants containing a known concentration (0.1 and 0.2 %) of aqueous extract. The aqueous extracts were applied together with adjuvant (Trend) for better adhesion to the leaf surface. Pepper plants with water adjuvant and 20 insects for each dish were used as the controls. The experiment was set up in three replicates and a control. The no-choice method, in which control and treated plants were placed individually in each dish, was adopted in this experiment. The mortality was checked after 24, 48 and 96 h.

Statistical analysis

Values of the biochemical parameters were expressed as means ± standard error (*SE*) of determinations made in triplicates and tested by ANOVA followed by comparison of the means by the Duncan multiple range test ($P < 0.05$). Data were analyzed using Statistica for Windows, version 11.0.

RESULTS AND DISCUSSION

Chemical composition

The total amount of phenols in *S. montana* aqueous extract was 53.96±3.9 mg GA equivalents g^{-1} dry weight. Flavonoids were found in an amount of 0.36±0.01 mg rutin equivalents g^{-1} d.w. According to Tahirović *et al*.[32], the total amount of phenols in *S. montana* herbal tea infusion was 78.5±23.49 mg GA 100 mL^{-1}. On the other hand, Chrpová *et al*.[24], found a lower amount of phenols (27.1 mg GA g^{-1}). The different amounts of the active substances found confirms that the chemical composition of the plants depends on the season in which they were collected, on the geographical area and other factors, such as environmental temperatures prevailing during plant growth.[33,34]

The main constituent of phenol components was caffeic acid (78.17 μg g^{-1}). The second largest component was gallic acid (15.36 μg g^{-1}). Quercetin, *p*-coumaric acid, chlorogenic acid and ferulic acid were represented at concentration of 2.36, 1.59, 1.36 and 0.50 μg g^{-1}, respectively. The HPLC-DAD chromatogram of standard solutions of the phenolic compounds prepared in mobile phase is shown in Fig. S-1 of the Supplementary material to this paper. Some of the validation parameters are given in Table S-I of the Supplementary material. The obtained *LOD* values for all the investigated phenolic compounds were 0.01 μg mL^{-1}, with *LOQ* values of 0.03 μg mL^{-1}.

The chemical composition study of *S. montana* aqueous extract enabled its properties to be understood, pending clarification of this work by a structure–activity relationship study.

Effect of extracts on MDA content and antioxidant enzyme activity in pepper and black nightshade seedlings

The response of plants to damaging adverse circumstances is closely related to their enzyme activity.[35] The obtained results showed a significant increase of peroxidases activities in the roots of black nightshade treated with 0.2 % *S. montana* aqueous extract 24 h after the treatment (Table S-II of the Supplementary material). The higher concentration had the same effect on the activity of CAT after 72 h. In the leaves of black nightshade, the activities of SOD and CAT were significantly increased by the lower concentration (0.1 %) of *S. montana* aqueous extract, while significant increases of the peroxidases activities were observed in plants treated with the higher concentration (0.2 %) 72 and 120 h after the treatment (Table S-III of the Supplementary material). The increases in the activities of SOD, CAT and peroxidases probably occur in response to stress.[35] Significant increases in lipid peroxidation were previously observed in various plant species under oxidative stress.[36] The malondialdehyde (MDA) content, an end-product of the lipid peroxidation process, is used as an oxidant biomarker. It was suggested that different concentrations of allelochemicals induce the production of

MDA; however, due to their antagonistic and synergistic effect, the MDA content is not always in reciprocity with the concentration of these substances.[36,37] In the roots of black nightshade, significant increases in the MDA content were recorded 72 and 120 h after the treatment (Table S-II). Two tested extract concentrations affected lipid peroxidation in the roots of black nightshade in the same way, but the higher level of MDA was observed in the roots treated with the higher concentration (0.2 %). The MDA content increased with the duration of the experiment. An increase in the MDA content indicates a high concentration of ROS that was beyond the threshold of scavenging by the antioxidant enzymes.[35] In spite of the increased activity of the enzymes, there were no significant changes in the LP intensity in the leaves of black nightshade between the plants from the control group and the treatments. This could indicate that the allelopathy-provoked stress was not strong enough and scavenging effects of SOD, CAT and the peroxidases could still prevent an oxidative burst and the induction of LP. The higher production of MDA in the roots of black nightshade compared with the MDA content in leaves showed that the roots were more affected by the allelochemicals than leaves. This may be attributed to the permeability of allelochemicals to root tissues arising from direct contact with the phytotoxic compounds present in the extract.[35] This finding is in agreement with the results of a previous study in which it was found that roots are more sensitive to allelopathic substances than shoots.[38]

In the roots and in the leaves of the pepper, there were no significant increases in the activities of CAT and peroxidases (Tables S-IV and S-V of the Supplementary material). An increase in the activity of SOD was detected after 120 h in plants treated with both concentrations of *S. montana* aqueous extract. In the leaves of pepper, no significant difference was recorded in the LP intensity. A significant increase in LP intensity was recorded 120 h after the treatment only in roots of pepper plants treated with the lower concentration (0.1 %).

The allelopathic effects of *Thymus kotschyanus* were studied on the growth of *Bromus tomentellus* and *Trifolium repens* seedling.[3] The phytotoxic effects of the extracts were found to be different between the two species under study, which points to different sensitivity of species when facing allelochemicals. Research on allelopathy of some plants of the Lamiaceae family on weeds showed that a water extract promoted growth of the test plants at low concentration, but inhibited them at high, and inhibition became stronger with increasing concentration.[39]

Toxicity test

Plant extracts are currently being studied as an ecologically friendly alternative to manage plant pests. Natural products have low mammalian toxicity as well as high target specificity and biodegradability, and contain many active ing-

redients, thus possessing biopesticide activity against multiple pests and pathogens.[40] In the present work, the aqueous extract of *S. montana* was evaluated on greenhouse whitefly. The mortality rate of greenhouse whiteflies after 96 h was above 50 % (Table I). The higher-concentrated *S. montana* aqueous extract (0.2 %) was more effective with a mortality of 68.33 %. The main constituent of the extract was caffeic acid, which was proved to have insecticidal activity.[41,42] According to some authors,[41] the presence of caffeic acid in *Impatiens parviflora* might be the reason for the insecticidal activity of this plant and the mortality of green peach aphid. Caffeic acid is one of the many phenolics considered as important parts of the defense mechanism of plants against microbial infection, insects and other predators.[42] The results of other authors indicate a potential use of aqueous plant extracts in pest management of greenhouse whiteflies.[43]

TABLE I. Mortality (%) of *Trialeurodes vaporariorum* adult fed for 4 days with formulations containing a known concentration (0.1 and 0.2 %) of *Satureja montana* aqueous extracts; a–d: values without the same superscripts within each column differ significantly ($P < 0.001$)

Formulation	Time, h		
	24	48	96
Control	8.33^{c}	10.00^{c}	16.66^{bc}
0.1 %	1.6^{d}	8.33^{b}	$41.66^{a,b}$
0.2 %	3.33^{d}	41.66^{a}	68.33^{a}

CONCLUSIONS

Extracts from different plant species and their active components are natural sources of biopesticides. This study contributes to an assessment of the potential use of medicinal plants as insecticides. The *S. montana* aqueous extracts were evaluated for their effect on greenhouse whitefly, an important insect pest of many plants, including pepper. It was observed that the aqueous extract with a concentration of 0.2 % showed a toxic effect with a high mortality rate 68.33 % after 96 h. The obtained results showed that use of natural substances could be an alternative method of insect control. This was supported by the results on the antioxidant properties of pepper seedlings. The extract did not exhibit any phytotoxic effect on the pepper or black nightshade seedlings. Therefore, it could be concluded that this plant should be explored in the development of bioinsecticides.

SUPPLEMENTARY MATERIAL

HPLC-DAD analysis and effects of the aqueous extracts of *Satureja montana* on the anti-oxidant enzymes and lipid peroxidation are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

АЛЕЛОПАТСКИ УТИЦАЈ И ИНСЕКТИЦИДНА АКТИВНОСТ ВОДЕНОГ ЕКСТРАКТА БИЉКЕ *Satureja montana* L.

ЈОВАНА ШУЋУР[1], АЛЕКСАНДРА ПОПОВИЋ[1], МИЛОШ ПЕТРОВИЋ[1], ГОРАН АНАЧКОВ[2], ВОЈИСЛАВА БУРСИЋ[1], БИЉАНА КИПРОВСКИ[1] и ДЕЈАН ПРВУЛОВИЋ[1]

[1]*Пољопривредни факултет, Универзитет у Новом Саду, Трг Доситеја Обрадовића 8, 21000 Нови Сад и* [2]*Природно–математички факултет, Универзитет у Новом Саду, Трг Доситеја Обрадовића 3, 21000 Нови Сад*

Широка употреба синтетичких инсектицида, хербицида и других пестицида има негативан утицај на околину, као и на здравље људи и животиња. Стога научници све више посвећују пажњу природним пестицидима, тј. активним компонентама биљних екстраката. У овом раду је испитан утицај две концентрације (0,1 и 0,2 %) воденог екстракта биљке *Satureja montana* L. на процес липидне пероксидације, и на активност антиоксидантних ензима у листу и корену паприке и црне помоћнице 24, 72 и 120 h након третмана. Добијени резултати су показали да 0,2 % концентрација воденог екстракта *S. montana* повећава интензитет липидне пероксидације у корену црне помоћнице. Поред тога, статистички значајно повећање активности пирогалол- и гвајакол-пероксидазе уочен је у листовима црне помоћнице након третмана са 0,2% воденим екстрактом *S. montana*. Други циљ рада био је испитивање токсичности воденог екстраката према лептирастој ваши. Водени екстракт концентрације 0,2 % проузроковао је смртност од 68,33 %.

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