Article

Phytotoxic Effects of Selected Herbal Extracts on the Germination, Growth and Metabolism of Mustard and Oilseed Rape

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Abstract: The use of plants that have high allelopathic potential as natural herbicides in the form of aqueous extracts is gaining popularity in environmentally friendly agriculture. Usually, their effect on the germination and growth of weeds is investigated. However, less attention is paid to the effect of the allelopathic compounds from extracts on cultivated plants. Therefore, the aim of this study was to evaluate the impact of herbal extracts that have allelopathic properties on selected physiological and biochemical processes of two plants of great economic importance—white mustard (*Sinapis alba* L.) and oilseed rape (*Brassica napus* L. var. *oleifera*). The extracts were prepared from mountain arnica (*Arnica montana* L.), ribwort (*Plantago lanceolata* L.), hypericum (*Hypericum perforatum* L.), common milfoil (*Achillea millefolium* L.), sunflower (*Helianthus annuus* L.) and sage (*Salvia officinalis* L.). The germination of white mustard and oilseed rape was most inhibited by the extracts that were prepared from sage and sunflower. Additionally, in the germinating plants, the sunflower extracts increased the membrane permeability, which indicates membrane injuries. The metabolic changes in the plants were monitored using isothermal calorimetry and FT-Raman spectroscopy. The total heat production, which provided information about the metabolic activity of the white mustard and oilseed rape, was decreased the most by the sage extract but generally all of the tested extracts disturbed the shape of the heat emission curves compared to the water control. The impact of the allelopathic compounds that are present in the herbal extracts on the metabolism of the seedlings was clearly visible on the FT-Raman spectra—in the fatty acids and flavonoids range, confirmed using a cluster analysis. In conclusion, the herbal extracts from medicinal plants that have herbicidal activity could be used as a natural herbicide for weed control, but since they may also have negative impacts on cultivated plants, preliminary tests are advisable to find the extract from the species that has the least negative effect on a protected crop.

Keywords: allelopathy; *Brassicaceae*; FT-Raman spectroscopy; isothermal calorimetry; natural herbicides

Citation: Rys, M.; Saja-Garbarz, D.; Skoczowski, A. Phytotoxic Effects of Selected Herbal Extracts on the Germination, Growth and Metabolism of Mustard and Oilseed Rape. *Agronomy* **2022**, *12*, 110. <https://doi.org/10.3390/agronomy12010110>

Academic Editors: Jeffrey Weidenhamer, Sajid Latif and Leslie A. Weston

Received: 29 October 2021

Accepted: 31 December 2021

Published: 2 January 2022

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1. Introduction

Modern agriculture is engaged in an ongoing fight against weeds. Weed interference with crops reduces yields and their quality [1] because they compete for water, nutrients and light. Additionally, they can cause enormous financial losses [2–4]. Weeds can be also the secondary hosts for some pests [5–7]. Therefore, synthetic herbicides are widely used to control weeds. However, continuously using synthetic herbicides can have a negative impact on human health and the environment. Using synthetic herbicides pollutes the soil and groundwater and enhances the toxic residue accumulation in agricultural products [8]. It can also lead to an increase in herbicidal resistance in many weed species [9].

In order to reduce the reliance on herbicides, integrated strategies are needed to improve weed management. Thus, there has been a clear increase in the interest of using

biological methods to protect plants against weeds—because they are environmentally friendly and inexpensive [10,11]. One of these is allelopathy, which refers to any direct or indirect effect of one plant on other plant through the release of chemical compounds (allelochemicals), which play an important role in agroecosystems, because they affect weeds as well as crops by producing allelochemicals [12]. These are released from donor plants (from leaves, flowers, seeds, stems and roots) as volatiles, foliage leachates, root exudates or through decomposed plant residues that, during the decay process or after being modified by microorganisms, are transformed and transported in the environment, reaching the acceptors [13–17]. Using allelopathy to control weeds also includes using allelopathic crop residues [18], cover crops [19], crop rotation [20], and catch crops [21], as well as selecting the allelopathic crop cultivars [22] and new herbicides that are derived from allelopathic chemicals [23]. Allelopathic compounds according to their different structures and properties can be classified into 10 categories: (1) straight chain alcohols, water-soluble organic acids, ketones and aliphatic aldehydes; (2) simple lactones; (3) polyacetylenes and long-chain fatty acids; (4) quinines (anthraquinones, benzoquinones, and complex quinines); (5) cinnamic acid and its derivatives; (6) phenolics (caffeic acid, coumaric acid, vanillic acid, syringic acid, ferulic acid); (7) coumarins; (8) tannins; (9) flavonoids; (10) terpenoids and steroids [24]. They could help to reduce the use of synthetic herbicides, which would lead to less pollution and safer agricultural products [25,26]. A large number of compounds have been identified as potential allelochemicals that could interact with the surrounding environment, such as phenolic acids, aromatic compounds, terpenes and flavonoids [27–29].

Nowadays, there is a clear increase in the interest in using plants that have an allelopathic potential (rich source of allelochemicals) as new natural herbicides [30–35]. Crop and herbal plants (well known for their medicinal properties) [36–38] offer potentially selective biological weed management by producing and releasing allelochemicals from the leaves, flowers, seeds, stems and roots of living or decomposing plant material [39,40].

Under natural conditions, the germination of seeds and the growth of plants occur in a soil solution with an extremely varied chemical composition (both in terms of the composition and the concentration of inorganic and organic substances). Of particular importance over the course of the growth processes is the presence of organic substances in the soil (deriving from, inter alia, the decomposition of plant tissues), which undoubtedly affects the physiological processes that occur in the germinating seeds and growing seedlings. As can be expected, the impact depends on the allelopathic potential of the compounds in the soil. In connection with this, allelopathy is also thought to be one of the indirect causes of continuous cropping obstacles in agriculture [41]. For a long time, most related studies have been conducted using compounds that have a high allelopathic potential and have focused on their effect on weeds [36,38,40]. Currently, more attention is paid to the correlation of natural substances with crops [42]. Therefore, as the tested species in our experiments, we selected the Brassicaceae family oilseed rape (*Brassica napus* L.) and white mustard (*Sinapis alba* L.), which are agronomically important crops that are harvested around the world and used for the production of oil, biofuel, and feed [43–45].

The species of herbal plants to be tested were selected based on a literature review. In the literature, the chemical compositions of several herbaceous plants were researched and those that contained many compounds that are commonly considered to be allelochemicals (phenolics, flavonoids, flavonols, anthocyanins, tannins, saponins, coumarins or alkaloids) were selected [46–50]. Six herbaceous plants that naturally occur in Poland were selected for the study. In addition, they very often appear in the fields among the crops of cereals and we wanted to check what effect they have on crops.

The aim of research was to evaluate the effect of herbal extracts that exhibit different allelopathic potentials—from herbs growing naturally in the fields among cultivated crops and that can be used as natural herbicides—on the germination, growth and selected metabolic changes of two important crop plants—mustard and oilseed rape. In the conducted research, two methods were used, which are rarely used in this type of investigation.

Isothermal calorimetry was used to determine the metabolic activity measured by the amount of specific thermal power emitted during the growth of seedlings. The changes in chemical composition induced in the storage organs by allelopathic compounds contained in the herbal extracts were investigated using Raman spectrometry with the Fourier transformation (FT-Raman). This technique allows study analytes in situ in their natural environment, what is very difficult using traditional analytical techniques. Additionally, it is a fast, non-invasive and non-destructive method.

The conducted experiments will determine the impact of allelopathic substances contained in aqueous herbal extracts, potentially used as natural herbicides against weeds, on cultivated crops among which these weeds grow. It may turn out that the negative effects on crops are as great as they are on weeds.

2. Materials and Methods

2.1. Plant Material and Preparation of Plant Water Extracts

Two species of economically important plants were selected for the experiments—white mustard (*Sinapis alba* L. cv. Nakielska derived by Rogowska's Plant Breeding in Dąbrówka Górna, Poland—50°543' N, 17°930' E) and oilseed rape (*Brassica napus* L. var. *oleifera* cv. Huzar derived by The Plant Breeding and Acclimatization Institute (IHAR), Strzelce, Poland—52°315' N, 19°406' E).

To prepare the plant (herbal) water extracts that have an allelopathic potential (which were applied to the white mustard and oilseed rape cultures), herbs—plants with a high concentration of active substances that have a documented healing effect—were selected. The following species were selected: flower of mountain arnica (*Arnica montana* L.), leaves of ribwort (plantain) (*Plantago lanceolata* L.), shoot (flowers, leaves and stem) of hypericum (*Hypericum perforatum* L.), shoot (flowers, leaves and stem) of common milfoil (*Achillea millefolium* L.) and leaves of common sage (*Salvia officinalis* L.). The dry plant material was purchased from “Herbapol—Lublin” S.A. (Poland). Moreover, the leaves of common sunflower (*Helianthus annuus* L. cv. Ogrodowy) were also used to prepare the extracts for testing, because it is a plant that has well described allelopathic properties [51–56]. All plants were harvested in the flowering phase.

The water extracts from the dry material were prepared by shaking 100 g of powdered plant parts in 1000 mL of distilled water (10% (*w/v*)) at room temperature for 24 h. The suspension was subsequently centrifuged (Universal 32R, Hettich, Germany) for 30 min at 22,000 × *g*. The supernatant was decanted and stored at −80 °C until it was used in the experiments.

2.2. Experimental Design

Two separate experiments were conducted within the study. In the first experiment, the effect of herbal extracts on the early stages of germination of oilseed rape and mustard was evaluated. The aim of the second experiment was to assess the growth and selected metabolic alterations in the young seedlings of white mustard and oilseed rape that had been exposed to an herbal extract with an allelopathic potential. All experiments were conducted in Krakow, Poland, 50°030' N, 19°550' E.

2.2.1. First Experiment

In experiment were used 140 Petri dishes (9 cm diameter) lined with filter paper. On each of 70 Petri dishes, 50 dry seeds of white mustard were placed and, on another 70 Petri dishes, 50 dry seeds of oilseed rape were placed. Then, 6 mL of each herbal water extract (arnica, ribwort, hypericum, milfoil, sage, sunflower) was added to 10 Petri dishes with white mustard or oilseed rape seeds (each extract on a different dish with seeds). Seeds that had been treated with water were used as the control. The Petri dishes were placed in an incubator at 20 °C in which the seeds were germinated in darkness. The number of germinated seeds was monitored during the seven days of the culture. One Petri dish is

one biological replicate, so for each tested species and herbal extract treatment and control, the experiment was repeated ten times, considered as ten biological replicates ($n = 10$).

2.2.2. Second Experiment

Fifty dry seeds of the tested species (white mustard and oilseed rape) were placed on a Petri dish that was lined with filter paper. Next, 6 mL of water was added and they were placed in an incubator at 20 °C in darkness. After 24 h of germination, 20 seedlings of a uniform size were selected and transferred to a new Petri dish that was lined with filter paper and then moistened with 5 mL of herbal extract or water (as a control). The dishes were placed in darkness at 20 °C for the next 48 h. The plant material that was prepared in this way (three-day-old seedlings) was collected to evaluate the effect of the herbal extracts on the growth of the seedlings, cell membrane permeability (conductometric measurements) and chemical composition (FT-Raman measurements). Only the one-day-old plants that had been germinated on water were used for the calorimetric measurements of metabolic activity. In this case, the herbal extracts were applied directly during a measurement (in a measuring ampoule—see Section 2.3.4). The number of biological replicates was given in the description of each analysis (Sections 2.3.2–2.3.5).

2.3. Measurements and Observations

2.3.1. Seed Germination

The plant material was prepared as described in Section 2.2.1. The seeds that germinated were counted once every 24 h from the first to the seventh day of the culture. The impact of the six herbal extracts on the germination of the white mustard and oilseed rape seeds was evaluated based on the number of germinated seeds compared to the control (seeds that had been germinated on water). The results are expressed as the percentage of the germinated seeds relative to the total number of seeds in a dish (per unit time). For each treatment (herbal extract), ten different Petri dishes with fifty seeds (white mustard or oilseed rape) on each dish were analysed. One Petri dish is considered as one biological replicate ($n = 10$).

2.3.2. Estimating the Growth of the Young Seedlings

The plant material was prepared as described in Section 2.2.2. The three-day-old seedlings were collected and then frozen at -80 °C, lyophilised (Freeze Dry System/Freezone 4.5—Labconco, Kansas City, MO, USA) and weighed in order to determine their dry weight. The impact of the herbal extracts on the dry mass of white mustard and oilseed rape was evaluated compared to the results obtained for the control seedlings cultured on water. For each herbal extract, twenty different Petri dishes with twenty seedlings (white mustard or oilseed rape) on each dish were analysed. One Petri dish is considered as a one biological replicate; twenty independent biological replicates were performed for each treatment ($n = 20$).

2.3.3. Plasmatic Membrane Permeability of the Seedlings

The plant material was prepared as described in Section 2.2.2. The conductometric measurements were conducted according to [57]. The three-day-old seedlings (twenty seedlings from one Petri dish) were first washed with deionised water and then placed in polypropylene vials in 8 mL of deionised water with a basal conductivity of $0.067 \mu\text{S}\cdot\text{cm}^{-1}$ and shaken gently for three hours, after which the electroconductivity of the diffusates was measured (L_t) (Conductometer CC 411, Elmetron, Zabrze, Poland). Then, the plant material was frozen at -80 °C in order to macerate the tissue. After 24 h, the material was defrosted, shaken for three hours and the electroconductivity was remeasured to determine the total content of electrolytes in the tissue (L_k). The measurements were performed in ten biological replicates for each treatment and control (tested species and herbal extracts). One biological replicate was one vial with twenty seedlings ($n = 10$). The discharge of

electrolytes was calculated as the percentage of the total amount of tissue using the formula: $EL = (L_t \cdot L_k^{-1}) 100\%$.

2.3.4. Calorimetric Measurements of the Metabolic Activity of Seedlings

The calorimetric measurements were conducted using an isothermal calorimeter (TAM III, Thermo Activity Monitor, TA Instruments, New Castle, DE, USA) according to a modified method described in [58]. Ten one-day-old seedlings that had been germinated on water were placed into measuring ampoule on filter paper with 400 μL of an herbal extract or water (control). The reference ampoule contained filter paper with 400 μL of a herbal extract or water (control). The stabilisation in the upper position was carried out for 30 min and once again after leaving the ampoules for another 30 min. The heat (specific thermal power) that was emitted by the growing seedlings was measured for 24 h at 20 °C. The measurements for each herbal extract and the control for white mustard and oilseed rape were taken in ten biological repetitions (one biological repetition was the measurement of ten seedlings in ampoule) ($n = 10$).

2.3.5. FT-Raman Measurements of the Chemical Composition of the Cotyledons

The three-day-old seedlings, which were prepared as is described in Section 2.2.2, were frozen at $-80\text{ }^{\circ}\text{C}$ and lyophilised (Freeze Dry System/Freezone 4.5—Labconco, Kansas City, MO, USA). An FT-Raman spectrometer was used to measure the chemical compositions of the seedling cotyledons (Nicolet NXR 9650, Thermo Fisher Scientific, Waltham, MA, USA). The spectrometer was equipped with an Nd:YAG³⁺ laser. The measurements were taken at room temperature with a laser power of 0.5 W, an aperture of 80 with a spectral resolution of 4 cm^{-1} in the range from 100 to 4000 cm^{-1} according to the method described in [59]. The measurements were taken in ten biological replicates for each tested species and treatment (herbal extract or water control) and then averaged. One biological replicate was the measurement of one cotyledon from one independent plant. The FT-Raman spectra were accumulated from 64 scans and registered and compiled using Thermo Scientific Omnic Software and Statistica 13.3. (StatSoft, Tulsa, OK, USA).

2.4. Statistical Analysis

Statistical differences of measuring parameters—seed germination, dry weight of seedlings, discharge of electrolytes and total thermal energy—between control and sample groups were analysed using the one-way ANOVA model. Statistical significance was set at $p \leq 0.05$ and were calculated using the Duncan multiple range test (Statistica 13.3. StatSoft, Tulsa, OK, USA). The number of repetitions is given above a particular method/measurement in each description. There is also a brief description of the statistical analysis in the caption under each figure.

A hierarchical cluster analysis of cotyledons (chemometry) was performed based on the obtained FT-Raman spectra within the range of $772\text{--}1680\text{ cm}^{-1}$ for both the white mustard and oilseed rape cotyledons using the Opus/Bruker Package computer program. The dendrograms were obtained using Ward's algorithm.

3. Results and Discussion

3.1. The Impact of the Herbal Extracts on the Germination of White Mustard and Oilseed Rape

The dynamics of the germination of white mustard and oilseed rape, which are expressed as the percentage of seeds germinated in time (one to seven days) on the herbal extracts and the control, are presented in Figure 1A,B. In the case of white mustard, the percentage of germinated seeds on the extracts of arnica, ribwort, milfoil, sunflower and sage did not exceed 20% until the fourth day, and even on the seventh day, it did not exceed 55%. Only the seeds on the hypericum extract that had already been germinated was it almost 60% on the second day, and it did not exceed 80% until the end of the experiment (day 7) (Figure 1A). In the case of oilseed rape, the highest germination rate was observed in the first two days. On the seventh day, the percentage of germinated seeds ranged from

approximately 90% to 100% in almost all of the extracts. The sage extract, which had an inhibitory effect and for which the number of germinated seeds did not exceed 70%, was the exception (Figure 1B).

Figure 1. Percentage of the seeds of white mustard (A) and oilseed rape (B) that had been germinated on the 10% (*w/v*) extracts from arnica (blue line), ribwort (green line), hypericum (red line), milfoil (pink line), sunflower (violet line), sage (light blue line) and water—control (black line). The mean values $\pm$ SD indicated by the same letters did not differ significantly at $p \leq 0.05$ according to Duncan's test (within the day of germination), $n = 10$.

The plant extracts that were used in the experiments contain many groups of allelopathic compounds—phenolic acids, flavonoids, terpenoids, lactons [46–55]—which have inhibitory activity [38,60,61]. These compounds might disrupt physiological and biochemical processes during seed germination. In the early stages of germination, the processes

of decomposition of storage materials accumulated in seeds usually proceed very rapidly, and in the case of oilseeds, this leads to lipid degradation. In seeds of mustard and oilseed rape germinating in the presence of phytotoxins from herbal extracts, a decrease in the degradation rate of storage lipids was found, which was probably caused by a decrease in the activity of lipolytic enzymes (Figure 1A,B). The nature of the effects of the extracts depends on various factors, including the allelopathic potential of the extract that is used, the treated species and perhaps also the storage material of the seeds that are exposed to the extracts. In our experiment, although both white mustard and oilseed rape belong to the same family (*Brassicaceae*) and their storage materials have similar chemical compositions, there were substantial differences in their ability to germinate in the same plant extract (Figure 1A,B and Figure 2A–N). Generally, oilseed rape seemed to be more tolerant to the treatment with the used extracts than white mustard. The inhibited germination of oilseed rape was observed only on the sage extract and is statistically different from the values (percent of germinated seeds) obtained on the other extracts. In the case of the remaining plant extracts, more than 80% of the seeds germinated at the end of the experiment. Finally, on the seventh day of the culture for oilseed rape, there was a higher percentage of seeds that had germinated on all of the extracts than in the case of mustard. Our results indicate that the hypericum extract had the least impact on germination and was therefore somehow the least toxic to both species. Germination was inhibited the most by the sunflower (mustard) and sage (oilseed rape) extracts—with statistically significant differences. However, in the case of white mustard, there was also a strong inhibition of germination for the sage and ribwort extracts. According to Kupidłowska et al. and Oracz et al., the inhibition of white mustard seed germination, which is induced by phytotoxins from a 10% sunflower leaf extract, results from the disruption of the typical metabolic processes in cells [56,62]. For example, they lead to the accumulation of reactive oxygen species, which caused cell damage and resulted in a decrease in germination capacity and a gradual loss of seeds' vigour. In our case, it can be assumed that the different reactions to the seed germination rate could have also resulted from other differences in the composition of the species other than their fat storage material.

Other studies also indicated the low allelopathic potential of milfoil extract. The germination of *Ch. album* seeds in the presence of this extract was not significantly inhibited [40]. On the other hand, the phytotoxic effect of sage on the germination of weed seeds and cereals was confirmed [63].

3.2. The Impact of the Herbal Extracts on the Growth of White Mustard and Oilseed Rape

Interestingly, the impact of the herbal extract on the seedlings that had previously been germinated on water was a different matter. This was especially visible in the case of oilseed rape but also partly in the case of white mustard. For white mustard seedlings grown on sage extract, the lowest dry mass accumulation value was found. Similar values of dry weight were found in control seedlings and seedlings growing on milfoil extract. There were no statistically significant differences between them. The seedlings growing on hypericum extract had the highest dry mass accumulation and they statistically significantly differed from all the others. The intermediate values of dry mass accumulation were shown by the seedlings growing on arnica, ribwort and sunflower extracts (Figure 3A). For oilseed rape, all of the herbal extracts caused a higher dry mass accumulation compared to the water control. The seedlings that were growing in only water (control) had the lowest dry mass accumulation (weight) and they statistically significantly differed from all the others. There was a statistically significantly high dry mass accumulation for the oilseed rape seedlings that had been exposed to the ribwort and milfoil extracts. The intermediate values of dry mass accumulation were shown by the seedlings growing on arnica, sage and sunflower extracts (Figure 3B).

Figure 2. Germination of white mustard seeds on the 10% (*w/v*) extracts of arnica (A), ribwort (B), hypericum (C), milfoil (D), sunflower (E) and sage (F). The white mustard seeds of the control (G) were cultured only in water. Germination of the oilseed rape seeds on the 10% (*w/v*) extracts of arnica (H), ribwort (I), hypericum (J), milfoil (K), sunflower (L) and sage (M). The oilseed rape seeds of the control (N) were cultured only in water. The photos are of the seven-day-old cultures.

In contrast to the biomass accumulation, as is visible in Figure 4, the lengths of plant sprouts were also different. The longest sprouts were observed in the control (for both white mustard and oilseed rape). The lowest dry weight was simultaneously noted in the control seedlings, which may confirm the assumption that germination and growth in distilled water that is undisturbed by stress factors permits the fastest decomposition of material in seedlings that are autotrophic at this stage of ontogenesis. The plant extracts also inhibited the loss of seedling weight somewhat (Figure 3A,B).

Figure 3. Dry weight of the three-day-old seedlings of white mustard (A) and oilseed rape (B) that were growing on the 10% (*w/v*) extracts from arnica, ribwort, hypericum, milfoil, sunflower, sage and water (control). The mean values $\pm$ SD indicated by the same letters did not differ significantly at $p \leq 0.05$ according to the Duncan's test (within the tested species), $n = 20$.

Because the observed changes in dry weight did not reflect the visual variation in the size of the seedlings, the dry weight parameter cannot be a good indicator for determining the impact of allelopaths on plant metabolism unless we incorrectly conclude that the higher accumulation of biomass in the treated plants is an indicator of the negative (growth inhibitory) effects of allelopathics. On the other hand, we have to remember that under natural conditions, the reduction in plant growth that is observed from allelopathic effects may be caused by the competition among plants for water, minerals, oxygen or space for their root system. Other factors that contribute to limiting growth are changes in pH or the osmotic potential of the soil, which is caused by the decomposition of plant residues. There are many influencing factors, which is why most of the information regarding allelopathy still comes from studies that are based on biological assays (bioassays) [40,64,65].

Figure 4. White mustard seedlings that were growing on the extracts of arnica (A), ribwort (B), hypericum (C), milfoil (D), sunflower (E) and sage (F). Control (G)—mustard seedlings cultured only in water. Oilseed rape seedlings that were growing on arnica (H), ribwort (I), hypericum (J), milfoil (K), sunflower (L) and sage (M). Control seedlings of oilseed rape (N) were growing only in water. The plants that were exposed to the extracts were first germinated in water for 24 h and then were cultured in the extract for 48 h. Photos show three-day-old plants.

3.3. The Impact of Herbal Extracts on Cell Membrane Permeability in White Mustard and Oilseed Rape

The sunflower, arnica and ribwort extracts increased the permeability of the cell membranes of the white mustard seedlings compared to the control (Figure 5A) and the values of electrolyte leakage fluctuated at approximately 20%; these differences were statistically significant. In contrast, for the seedlings that were growing in the hypericum extract, the electrolyte leakage values were lower than those of the control seedlings and statistically significant from all other extracts (including control) (Figure 5A). The permeability of the cell membranes in the oilseed rape seedlings increased substantially under the influence of all of the plant extracts compared to the control. The values of electrolyte leakage were the lowest (about 13%) and statistically significant for control seedlings compared to the values for seedlings growing on all herbal extracts. The highest and statistically significant increase, up to approximately 30%, was caused by the sunflower, ribwort and sage extracts (Figure 5B).

Figure 5. Membrane permeability expressed as changes in the electrolyte leakage of the white mustard (A) and oilseed rape (B) seedlings that were growing in the presence of the extracts from arnica, ribwort, hypericum, milfoil, sunflower, sage and water (control). Mean values $\pm$ SD indicated by the same letters did not differ significantly at $p \leq 0.05$ according to the Duncan's test (within the tested species), $n = 10$.

Stressful environmental conditions very often disturb the permeability of membranes (usually measured as electrolyte leakage), which makes it a sensitive indicator of plant membrane injuries [66–68]. The increased outflow of ions and metabolites occurs both during the early and later stages of plant exposure to biotic and abiotic environmental stresses. Stress factors may condition the discharge of ions via chemical changes in the composition of the membranes or the phase transitions that occur in the membranes [69]. The plant extracts in which the seedlings were growing generally increased the membrane permeability and, undoubtedly, this phenomenon caused a disruption in the water and mineral management in the seedlings. The effect on the cytoplasmic membranes, however, is the primary source of disruption and their injuries result in the entire metabolic system of a plant becoming unbalanced. It is assumed that in the broad spectrum that is formed by the allelochemical compounds, the permeability of membranes is affected by the primary phenols. Einhellig [70] assumed that the molecules of allelocompounds penetrate the membrane matrix and either destroy their structural links or create new ones. However, they may also modify the channels that exist in membranes, which leads to a disruption in ion transport. Additionally, as already mentioned, allelopathic compounds cause the accumulation of reactive oxygen species and free radicals. This is the reason for lipid peroxidation leading to the degradation of polyunsaturated fatty acids (PUFA). It is likely that lipid peroxidation degrades PUFAs present in membranes, leading to their damage [71,72]. It can also lead to an oilseed lipid reserve. According to [62], lipid peroxidation occurs when the seed begins to die and therefore it can be assumed that deep damage to the lipid

component of the seed also occurs, which may be related to the membrane or reserve lipids. Our experiments showed that the hypericum extract resulted in the least significant changes to the permeability of the cell membranes of the white mustard seedlings (Figure 5A). In oilseed rape, the slightest membrane injuries were on the arnica extract, while the hypericum extract also caused minor membrane injuries (Figure 5B). The strongest effect on the increased permeability of the cell membranes and statistically significant differences for both tested species was observed for the sunflower extract (Figure 5A,B). This indicates that the sunflower extract contains particularly large quantities of the chemical substances that are damaging to the functioning of the cell membranes, which correlates well with our results described above, where the same extracts had the least significant (hypericum) and the most significant (sunflower) inhibiting effects on the germination of seeds (Figure 1).

3.4. Specific Thermal Power Curves and Amount of Total Heat in the White Mustard and Oilseed Rape Seedlings That Had Been Exposed to the Herbal Extracts (Metabolic Activity of the Seedlings)

It is assumed that the thermal power that is emitted by plant tissue and the total amount of energy that is emitted are directly proportional to its metabolic activity [73].

In the control plants of white mustard (Figure 6A) and oilseed rape (Figure 6B) that were growing in only water, the obtained curves of the specific thermal power were linear with a tendency to increase in time from 0 to 24 h. The values of the heat emission that was registered for the control white mustard and oilseed rape seedlings at the start of the measurement were 2 and 3 mW·gDW^{−1}, respectively, and after 24 h, they were 14 and 10.5 mW·gDW^{−1}, respectively. In contrast to the control, the shape of the curves of the specific thermal power that was obtained for the plants that were cultured on the individual extracts was highly diverse (Figure 6A,B).

For the curve for the specific thermal power, two or three areas could be distinguished. The first one was where there was an increase in the emissions of thermal power (strong exothermic effect). The second one illustrated a rapid drop that often transformed into a strong endothermic effect (negative values of thermal power) and re-growth. Only the curves obtained during the growth of the seedlings in the hypericum extract differed substantially from the curves that were obtained for the other extracts. Endothermic effects (a rapid decline in thermal power) for the white mustard seedlings occurred during the growth in the arnica extract (Figure 6A). In the case of oilseed rape seedling growth, the curves of the specific heat capacity had negative values in practically all of the extracts apart from the hypericum extract (Figure 6B). However, these negative values were primarily observed during the growth in the arnica, ribwort and milfoil extracts. The timing of the individual peaks strongly depended on the type of extract. The maximum exothermic peak was observed in the white mustard seedlings at a similar time in the case of the milfoil and sage extracts (after approximately six hours). The ribwort and sunflower extracts peaked after more than ten hours (Figure 6A). The endothermic peak only appeared in the arnica extract after 21.5 h of seedling growth. In the case of the growth of the oilseed rape seedlings (Figure 6B) in the sage extracts, the maximum of the exothermic peak was observed after less than ten hours. The last one to be observed was the exothermic peak in the hypericum extract (after more than 13 h of growth), while, in the other extracts, these occurred at similar times. The endothermic peaks were observed first during the seedling growth in arnica and sage extracts (more than 16 h) and last in the sunflower extract (more than 22 h). No endothermic reactions were observed in the hypericum extracts.

Figure 6. The specific thermal power curves of the white mustard (A) and oilseed rape (B) seedlings that were growing on the extracts from arnica (blue line), ribwort (green line), hypericum (red line), milfoil (pink line), sunflower (violet line), sage (light blue line) and water (control) (black line). The mean values were based on ten repetitions. The total heat that was emitted by the white mustard (C) and oilseed rape (D) seedlings that were growing on the extracts from arnica, ribwort, hypericum, milfoil, sunflower, sage and water (control). Mean values $\pm$ SD indicated by the same letters did not differ significantly at $p \leq 0.05$ according to Duncan's test (within the tested species), $n = 10$.

The effect of herbal extracts on the overall metabolism of seedlings can be summarised and illustrated by the amount of total thermal energy (total heat) that is emitted during their growth (24 h), which is also known as the enthalpy of seedling growth. The value of the power is equal to the area under the curve of changes in the specific thermal energy over time and was calculated by integrating the curves shown in Figure 6A,B. As is shown in Figure 6C, the ribwort extract caused an increase ($\sim 555 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$) while the sage extract caused a decrease ($\sim 185 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$) in the total heat that was emitted by the white mustard seedlings compared to the control. The values of the total heat for the seedlings that were growing in the remaining extracts were statistically similar to the control—ranging between ~ 250 and $400 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$ (Figure 6C). For oilseed rape, the amount of total heat that was emitted during the growth of the control seedlings ($\sim 310 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$) did not differ statistically from the seedlings that were growing in the hypericum ($\sim 330 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$) and ribwort ($\sim 265 \text{ J} \cdot \text{g}_{\text{dw}}^{-1}$) extracts. For the sage extract, the amount of total heat that was emitted was the lowest (only $85 \text{ J} \cdot \text{g}_{\text{sm}}^{-1}$) (Figure 6D).

The vital processes that run in living organisms are characterised by the changes in the amount of energy. It is possible to observe these changes using isothermal calorimetry, which enables the impact of external factors during the process of seed germination and seedling growth to be monitored [59,74,75]. Isothermal calorimetry is also useful for

testing the allelopathic effects in plants [56,59,76–78], including the effects of essential oils on plants [79]. The thermal energy that is emitted reflects the viability of seeds and the response to stressors such as toxins including phytotoxins [80] or low temperatures [81]. We observed that the specific thermal power curve shapes for the seedlings that were growing in the plant extracts were different from the shapes of the curves obtained for the control samples (Figure 6A,B). Moreover, they were quite complex and impossible to describe with a simple mathematical function, as was the case for the control. This illustrates the strong influence of allelopathic substances on the metabolism of seedlings and the intensity and complexity of the physiological processes that are initiated by allelochemicals. Particular attention must be paid, however, to the similarity in the changes in the shape of the specific thermal power curves over the period in the seedlings of both species that were analysed that were growing in the same extract. Given the natural variability in the chemical composition of the tested extracts, this might suggest that plant metabolism is disturbed by substances that have similar chemical properties. Perhaps these substances belong to the same groups of compounds: phenols, glycosides and coumarins. The curves that were obtained during the growth of the seedlings in the hypericum extract differed substantially from the curves that were obtained for the other extracts. The hypericum extract had the slightest negative influence on the seed germination energy and plasmatic membrane permeability. The total values of thermal energy varied relative to the total thermal energy value for the control seedlings (Figure 6C,D). Nevertheless, one can see a clear pattern that consists of a decrease in the total thermal energy (relative to the control) for the sage and sunflower extracts. As indicated above, the extracts also inhibited the seed germination of the tested plant species, which may indicate that the extracts and, more specifically, the allelopathic substances they contain have the greatest inhibitory effect on the metabolic processes that occur during seed germination and seedling growth [59,77,78]. Furthermore, it is generally accepted that the growth of plants is an exothermic process, i.e., accompanied by heat emission into the environment. Although there have been references regarding the occurrence of endothermic effects during the research into seed germination [82], during the experiment this effect was observed during the growth of seedlings. The absorption of heat from the environment by the growing seedlings was even greater during the experiment in which both the seed germination and seedling growth occurred entirely in the plant extracts (excluding the germination in water) [76]. These effects were observable using only the isothermal calorimetry.

3.5. Analysis of the Changes and Similarities of the Chemical Composition of the White Mustard and Oilseed Rape Seedlings That Had Been Exposed to Herbal Extracts

The FT-Raman spectra showing the chemical composition of seedlings cotyledons are presented within the range of 700 to 1950 cm^{-1} for the white mustard (Figure 7A) and 1120 to 1760 cm^{-1} for oilseed rape (Figure 7B). The differences between the species arose from the different positions of the characteristic marker bands. The individual groups of the chemical compounds that are contained in plant tissues have specific marker bands that are unique to each group, which enable them to be identified with specific groups. Although they appear for all objects, they have different intensities (peak height). This was due to changes in the content of the analysed compound groups during seedling growth, which was affected by the chemicals that were present in the plant extracts that were used. The bands that are derived from the vibrations within the specific chemical bonds appear in the Raman spectra.

Figure 7. FT-Raman spectra showing the chemical composition of seedlings cotyledons of the white mustard (A) and oilseed rape (B) that were growing on herbal extracts: A—arnica (blue line), B—ribwort (green line), C—hypericum (red line), D—milfoil (pink line), E—sunflower (violet line), F—sage (light blue line), G—water (control) (black line), $n = 10$. Hierarchical cluster analysis of the FT-Raman spectra of the white mustard (C) and oilseed rape (D).

The most characteristic wavenumber range, which had several distinct bands, occurred between 1752 and 845 cm⁻¹. The prominent band at about 1440 cm⁻¹ and signals near 1654, 1302 and 1632, 1264 cm⁻¹ were assigned to the fatty acids. Additionally, a characteristic flavonoid band near 1593 cm⁻¹ was detected. The FT-Raman measurements enable the degree of the unsaturation of the fatty acids to be investigated. The ratio of the scattering intensity that arises from the C = C stretching vibration (1600 cm⁻¹) to the one that is obtained from the CH₂ scissoring mode (1444 cm⁻¹) can be used to reliably predict the iodine values of the unconjugated vegetable oils. The total degree of unsaturation can also be determined by calculating the ratio of the intensity of the band at ~1650 cm⁻¹ to that of the bands at ~1630 cm⁻¹ and ~1270 cm⁻¹ to that of the band at ~1300 cm⁻¹ [83,84]. The most significant difference between the spectra was observed between the seedlings that were growing on water (control) and those that were growing the various herbal extracts (Figure 7A,B). This is mainly related to the ratios of the 1654, 1632 cm⁻¹ and 1270, 1300 cm⁻¹ bands, which can be correlated to the degree of the unsaturation of the fatty acids in these samples as well as with the content of flavonoids.

The comparative analysis of the chemical composition of white mustard and oilseed rape seedlings that had been exposed to the herbal extracts was conducted using FT-Raman spectroscopy. The obtained spectra indicate that the herbal extracts affected the chemical composition of the embryonic leaves of the seedlings. In the cotyledons of the white

mustard and oilseed rape, the bands that were derived from the saturated and unsaturated fatty acids differed in height. The plant extracts that were used, therefore, had a strong impact on the changes in the degree of the unsaturation of lipids. Accordingly, a disorder in the proportions between the saturated and unsaturated fatty acids occurs, which may result, for instance, in changes in the physical properties of the cell membranes that are formed (fluidity and/or permeability). The Raman spectrometry showed the proportions between the saturated and unsaturated fatty acids that were caused by the allelopathic substances contained in the plant extracts, which were clearly indicated in the obtained spectra [59,78]. At the early stages of germination, the decomposition of the storage material that had been collected in seeds generally proceeds quite rapidly, and, in the case of oilseed rape, it led to the degradation of lipids, which are the main source of carbon for growing seedlings. These effects were also observed in studies of lima bean [85], peanuts [86] or white mustard [87]. The lipid content is dependent on both the rate of the processes of their synthesis and their use to produce other compounds. In the germinating rape seeds, there was a decrease in the degradation rate of the lipids in presence of phytotoxins, which was probably caused by a decreased activity of the lipolytic enzymes. It has also been found that the inhibition of auxiliary lipid mobilisation results in the lack of an embryo cell's ability to grow expansively. This, in turn, leads to a halted process of seed germination, which is one of the most common effects of phytotoxic substances [56].

In addition, distinct changes were found in the content of the flavonoid compounds in the cotyledons. If we assume that flavonoid compounds are an important element of the antioxidant system in a cell [88], then allelopaths either significantly reduce its ability to scavenge free radicals (decreased quantity of flavonoids) or cause oxidative stress, which triggers the cell's defence mechanisms (resulting in an increased synthesis of flavonoids).

Hierarchical cluster analysis (similarity analysis) is a kind of statistical method that classifies tested objects into groups (clusters). As a result, the obtained clusters are objects that are as similar as possible. Cluster analysis is used to find meaningful and systematic differences among the measured FT-Raman spectra on which specific groups of the chemical compounds present in the plant tissues are visible.

Cluster analysis was conducted to compare the differences in the chemical composition of the analysed species and was performed in the range from 772 to 1680 cm^{-1} for the cotyledons of the white mustard and oilseed rape seedlings (Figures 7C and 7D, respectively). For mustard, three distinct groups were observed. The first group included the seedlings that had been exposed to the arnica, ribwort and hypericum extracts. The second group was included the seedlings that were growing in the milfoil, sage and sunflower extracts. The third, and completely separate group, was composed of the control seedlings, which indicated significant differences in the chemical composition of the samples that were growing in distilled water compared to those that were growing in the herbal extracts. The cluster analysis for cotyledons of the oilseed rape seedlings (Figure 7D) identified two main groups, between which the differences were statistically significant. The first included the seedlings that had been exposed to the arnica, ribwort, hypericum and sunflower extracts, and the second included the seedlings that were growing in the hypericum and sage extracts and control samples.

Based on the cluster analysis, attempts were made to group the species according to the similarity of the impact of a plant extract on the chemical composition of the cotyledons. The cluster analysis performed for the cotyledons of the white mustard seedlings showed that the control samples differed in their chemical compositions from all of the others, but mostly from those that were growing in the arnica and ribwort extracts. For the oilseed rape cotyledons, the most distant objects were the cotyledons of seedlings that were growing in the arnica extract (to a lesser extent in the ribwort) and control. Thus, it can be concluded that these are the extracts that had the greatest impact on the metabolic changes that were observed in the cotyledons of oilseed rape.

4. Conclusions

This study demonstrated that all of the tested herbal extracts have allelopathic potential in terms of seed germination, seedlings growth, and the metabolism of white mustard and oilseed rape. The extracts from the sunflower and sage leaves strongly inhibited seed germination and the growth of the seedlings. The least impact on the metabolism of the tested species compared to the control was observed for the hypericum extract. The strong phytotoxicity of the tested extracts, which could be used as natural herbicides, requires further research using a wider variety of crops and weeds. Moreover, since the herbal extracts are not neutral to cultivated plants, preliminary tests would always be advisable to enable the extract of the lowest negative effect on protected crops and the highest on weeds to be selected. Further studies (also in field conditions) would also enable the more precise selection of the dose and method of application of allelopathic compounds in order to minimise weed infestations without changing (or only to a minimal extent) the metabolism of cultivated crops. Given the promising results of sage and sunflower extracts, they could be used for further research to develop a natural herbicide that is environmentally friendly.

Author Contributions: M.R.—conceptualising and designing the experiments, analysing the FT-Raman spectroscopy, measuring the electrolyte leakage as well as analysing the seeds germination and seedlings dry weight, calculating the data and performing the statistical analyses, preparing the figures, writing the manuscript; M.R. and D.S.-G.—analysing the calorimetry measurements; A.S.—conceptualising and designing the experiments, supervising, taking photos, interpreting the data, critically reading and correcting the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank Anna Janeczko for her valuable suggestions when writing this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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