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Author(s): Svenberg, Linus; Emmer, Åsa

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Manuscript Abstract for ed-2022-00254m:

The understanding of analytical chemistry and its application in different areas of life science, such as chemical ecology, is something that should be encouraged for students studying chemistry, and students interested in above mentioned areas. It is therefore beneficial to offer students inquiry-based project work that allows them to explore, and gain a deeper understanding of, this discipline within the grand context that is chemistry. Hence, the present work describes the application of analytical chemistry using gas chromatography – mass spectrometry, including sample preparation for the study of allelopathic properties of compounds found in the plant Aegopodium podagraria. The questions posed to the students performing this project work were “What compounds can be found in the extracts of the plant?”, “Are the extracts allelopathic?” and “Can you determine which compounds contribute to the possible allelopathic properties?”. The reported results indicate that the extracts have allelopathic properties and showed that the extracts contained compounds such as α-pinene and β-caryophyllene. The identified terpenes were shown to have minor allelopathic properties by themselves but displayed an impact on the germination of seeds and length of the sprouts when applied as a blend. This project, and its results, proposes a framework for investigation of other plants, and can be adapted to suit students at different academic levels.

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Thank you.

Investigation of the ~~allelopathic~~ chemical inhibition effect of Ground Elder (*Aegopodium podagraria*) on Timothy (*Phleum pratense*) – Introducing high school students to analytical chemistry and chemical ecology

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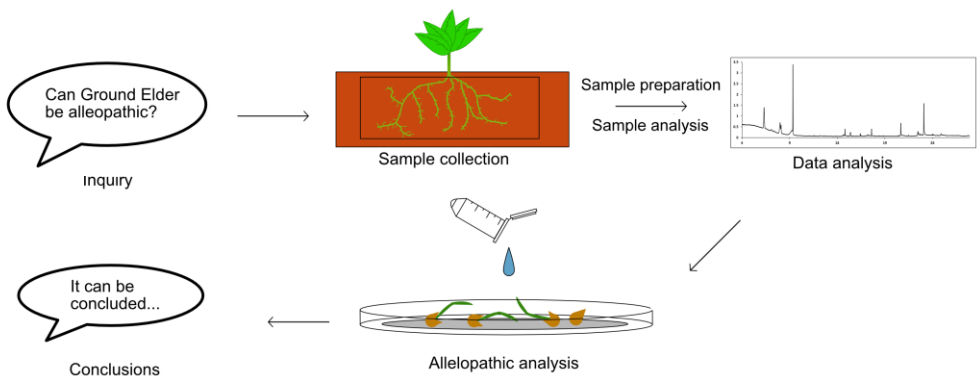
ABSTRACT

The understanding of analytical chemistry and its application in different areas of life science, ~~such as chemical ecology,~~ is something that should be encouraged for students studying chemistry, and also something that appeals to many students~~ and students interested in above mentioned areas~~. Life science includes a vast variety of areas, out of which some are more easily approached in student projects. The chemical communication between organisms, such as plants, as described in chemical ecology is one example. It is therefore beneficial to offer students inquiry-based project work that allows them to explore, and gain a deeper understanding of, this discipline within the grand context that is chemistry. Hence, the present work describes the application of analytical chemistry using gas chromatography – mass spectrometry, including sample preparation for the study of chemical inhibitory (allelopathic) properties of compounds found in the plant *Aegopodium podagraria*. The questions posed to the students performing this project work were "What compounds can be found in the extracts of the plant?", "Are the extracts allelopathic?" and "Can you determine which compounds contribute to the possible allelopathic properties?". The reported results indicate that the extracts have allelopathic properties, and showed that the extracts contained compounds such as α-pinene and β-caryophyllene. The identified terpenes were shown to have minor allelopathic properties by themselves but displayed an impact on the germination of seeds and length of the sprouts when applied as a blend. This project, and its results, proposes a framework for investigation of other plants, and can be adapted to suit students at different academic levels.

GRAPHICAL ABSTRACT



KEYWORDS

Chemical ecology, Analytical chemistry, Ultrasound Assisted Extraction, Gas-Chromatography, Mass-Spectrometry, Inquiry-based, Project based learning

BACKGROUND

Plants may seem to be defenseless organisms, exposed to many dangers and threats for their survival and reproduction. In reality, this is not always the case. There are innumerous examples on intricate means for plants to interact and communicate with their environment to secure their prosperity, even on the expense of other species. One way to communicate is by the use of chemicals. The science covering this and other types of chemical communication in nature, is called chemical ecology[1].

The scope of chemical ecology is vast, and includes allelopathy (chemical inhibition), which describes a plant's ability to germinate, while inhibiting the growth of other plants in its proximity, utilizing compounds called allomones[1]. Allomones fall into the category of substances (semiochemicals) that are used to signal to other organisms, changing their behavior or conditions of life [2]. Examples of semiochemicals with allelopathic properties are α-pinene[3], camphor[3,4], limonene[3] and β-caryophyllene[5], to name a few. Previous literature has described the investigation of allelopathy of individual compounds[6]. There has also been work performed with regards to identifying principal chemical components of plants and flowers[7] as exercises for students. Extraction of plants with allelopathic properties has been

demonstrated as a laboratory exercise appropriate for high school students[8], and analysis of volatiles with gas chromatography (GC) and mass spectrometry (MS) has been shown for undergraduates[9]. In this work, a combination of the previously mentioned exercises is demonstrated, including GC-MS analysis. It can be assumed that many students at the high school level are aware that plants emit odorous compounds. However, it cannot be assumed that they are aware of the fact that these odor compounds are used by the plants to interact with other plants in their surroundings. Thus, a study in allelopathy can offer high school students a deeper understanding, not just about allelopathic plant-plant interaction as an example of biological and chemical communication in nature, but also about the analytical methods that are used to isolate and identify these odorous compounds. In order to increase the interest and understanding of this branch of chemical ecology, a collaboration between the Royal Institute of Technology (KTH) and Blackebergs Gymnasium (BGYA), both situated in the area of Stockholm, Sweden, was initiated. The high school students involved were participating in the third year of the three-year Natural Science Program at BGYA, and were performing their diploma project (100 credits out of in total 2500 credits). They had taken 650 credits in natural science (chemistry, biology and physics) but had no prior experience of neither chemical ecology or GC-MS. The collaboration was performed as an inquiry-based project, as to allow the students to relate observations, such as the allelopathic strength of the extracts, to their self-generated data, which would be the GC-MS results[10]. In the case of this work, it can be claimed that the concept of structured inquiry is followed, as the students are presented with the problem and a framework of how to produce a solution[11]. The use of structured inquiry has shown to promote more reflection among the students regarding the improvement of their work, than obtained in conventional laboratory work that is more focused on verification[12]. This can, along with the writing of a report to aid in reflection on the quality of the study, further develop this project using the feedback of the students[13]. This project provided the high school students the chance to immerse themselves in the disciplines of analytical chemistry and chemical ecology. For the past five years, a project with the aim to investigate the allelopathic effect of the plant *Aegopodium podagraria*, commonly referred to as Ground Elder, has annually been offered to a group of three high school students from BGYA[14-18]. In previous years, an extraction method using ethyl acetate as extraction solvent, has been developed to produce extracts that showed allelopathic effects on seeds of among others *Phleum pretense*, otherwise known as Timothy. Abstracts of reports from previous years, including a summary of the iterative development of the experimental methodology, can be found in the Supporting Information (S1). The promising results reached through the years have now been used as the framework for the study performed by the students in this work (full student report in Supporting Information, S2). Previous years' work has proved the usefulness of the method for extraction combined with utilization of GC-MS for identification of potential allelopathic compounds from *e.g. A. podagraria*. The task

given to three students from BGYA this time included both to evaluate the allelopathic effect of the extracts obtained with a predetermined extraction method from the Ground Elder plant, and to identify the volatile organic compounds (VOCs) present in their samples using an existing GC-MS analysis method. The students should then select individual identified VOCs, based on availability in the lab, for trial in growth studies evaluating their allelopathic effect on the Timothy seeds. The present work describes the full method executed by the students, including Ultrasound Assisted Extraction (UAE), Solid-Phase Extraction (SPE) purification, GC-MS analysis and growth trials to determine the allelopathic effect of *A. podagraria.* Moreover, an overview of the results obtained as well as a student evaluation of the project, and a short discussion regarding possible improvements and adaptions of the project are included. The UAE, SPE and GC-MS analysis were performed at KTH, during in total about a full week, spread over a few occasions. The rest of the work, including growth studies, was performed at BGYA.

EXPERIMENTAL SECTION

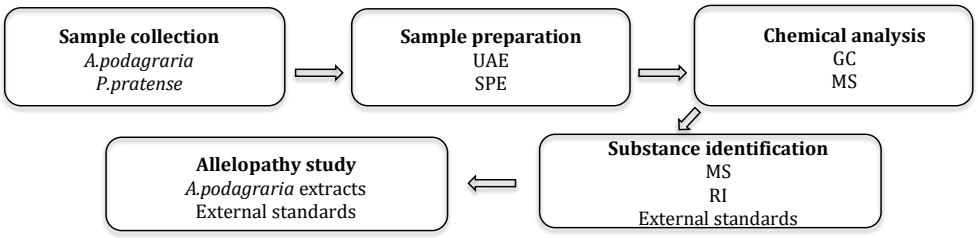


Figure 1: Schematics of the experimental process.

Sample Collection

Samples of *A. podagraria* were collected by the students at Råcksta träsk (59°21'05.0"N 17°52'41.2"E) and then brought to KTH the following day. The roots were washed using doubly distilled water (Milli-Q) from a Synergy 185 water purification system (Merck, Kenilworth, NJ, USA) with a resistivity of 18.2 MΩ*cm at 25 ˚C in order to remove any residual soil left on the plant samples. The samples were stored in a -80 ˚C freezer at KTH until further use. Generic photos of the plants can be found in Supporting Information S3.

Sample Preparation

One set of 5 replicates, and 1 control sample, for two different plant mass-to-solvent ratios were prepared for UAE at three separate occasions. For each occasion, this yielded 10 extracts from roots and 2 control extracts, resulting in a total of 30 samples and 6 control samples. Three rounds of extracts were prepared in order for the students to be able to generate a larger dataset in the growth study than had previously been obtained by other students. With the larger dataset, statistical properties such as mean and variance could

be calculated. Only the roots of the plants were used for the extractions, and the two plant mass-to-solvent ratios used for the sample preparation were 0.5 g/mL and 1 g/mL. After the plant mass had been placed in a Polypropylene (PP) tube and weighed, the appropriate volume of LC-grade acetonitrile (Merck Millipore, Burlington, MA, USA) was added to the tube. The samples were allowed to soak for 30 minutes prior to extraction, after which they were placed in a Sonorex RK100H (Bandelin, Berlin, Germany) ultrasonic bath for 60 minutes, at room temperature. The ultrasonic bath had a peak output of 320 W, and no additional heating was applied during the extraction. After extraction the solvent was removed from the falcon tube and filtered through a 0.2 µm polypropylene filter.

The extraction samples were then purified, and the analytes concentrated using Bond Elut (Agilent Technologies, Santa Clara, CA, USA) SPE cartridges with a C18 stationary phase, particle size of 40 µm, 200 mg bed mass and 3 mL cartridge volume. The solvents used had the following grades: GC-grade ethyl acetate (Sigma Aldrich, St. Louis, MO, USA), GC-grade hexane (Sigma Aldrich) and LC-grade acetonitrile (Sigma Aldrich). For each sample, a cartridge was washed with 3 mL of a 1:3 volumetric ratio solution of ethyl acetate and hexane and then conditioned with 3 mL of acetonitrile. The sample was then loaded onto the cartridge bed, after which the bed was eluted once with 1 mL of a 1:8 volumetric ratio solution of ethyl acetate and acetonitrile and then eluted a second time with 1 mL of a 1:3 volumetric ratio solution of ethyl acetate and hexane. Both of the 1 mL fractions, labelled Elution I and Elution II chronologically, were collected for GC-MS analysis and allelopathic trials. Prior to GC-MS analysis, 6 µl of an 8.62 mg/mL solution of heptyl acetate (Sigma Aldrich) in hexane was added to 594 µl of each sample as internal standard, resulting in 86.2 µg of internal standard in every sample. Hexane blanks for analysis were also prepared using the sample preparation method. These blanks were also used as control samples in the allelopathic studies of the extracts.

The sample preparation was performed by the students at KTH, but the parameters listed above (with some minor modifications) have been used in earlier applications[19]. There are still room for improvement, or adaption to other plant material, though.

Gas-Chromatography Mass Spectrometry Analysis

A 7890A GC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a 30 m x 0.25 mm x 0.25 µm DB-5MS column (Agilent Technologies), coupled to a 5975C mass spectrometric detector (Agilent Technologies) was used for all analyses. The GC oven was programmed to execute a 28 minutes long temperature program starting at 40 ° C, maintaining this temperature for 1 minute, followed by a temperature ramp of 10 ° C/min until a temperature of 260° C was reached, which then was held for 5 minutes. A Cooled Injection System (CIS) (Gerstel, Mülheim an der Ruhr, Germany) was used as injection

port, and was programmed to execute a temperature program starting at an initial temperature of 40° C for 6 seconds, followed by a temperature ramp of 12° C/s until the injector port reached a temperature of 260° C to finally hold this temperature for 2 minutes. The ion source temperature was set to 230° C, the mass range was set to 35 - 425 m/z, electron ionization was set to 70 eV, and a solvent vent was applied for the first 4 minutes of each analysis. LAB-LINE Helium 6.0 (Strandmöllen AB, Ljungby, Sweden) was used as a carrier gas and a MultiPurpose Sampler (MPS) (Gerstel) was used to inject 1 µl of sample per injection automatically. All GC-MS analyses were performed in the lab at KTH by the students with the assistance of one of the authors (L. Svenberg). As was the case for the sample preparation, the parameters listed above (with some minor modifications) have been used in earlier applications[19].

Peak detection was performed using the integration function in Chemstation (Agilent Technologies) data analysis software with the integration events set as follows; initial area reject was set to 1500000, initial peak width was set to 0.02, shoulder detection was set to off, and initial threshold was set to 15.0. This was done in order to get as consistent integration as possible between runs, avoiding manual integration performed by inexperienced students. Identification using mass spectrometry was done by comparison of recorded mass spectra to that of the National Institute of Standards and Technology (NIST)/Environmental Protection Agency (EPA)/National Institute of Health (NIH) Mass Spectral Library version 2.0 g, build 2009. Retention indices were calculated by comparison of retention times to that of the 49452-U C_7-C_{40} alkane standard (Supelco, Bellefonte, PA, USA) (10 µg of each in hexane). In addition, external standard aided identification was done by comparing sample analyte peak retention times to that of the retention times of the external standards 268070 α-pinene (Sigma Aldrich) and 22075 caryophyllene (Sigma Aldrich).

During the sample analysis, samples were randomly selected to be analysed in triplicate as to show the robustness of the GC-MS method. The non-selected samples were analysed once in the sake of time available. The distribution of the analyses is shown in the Supporting Information, in table 1S.

Allelopathy Study

Growth studies were performed at BGYA by the students. The trials were conducted in petri-dishes with filter paper placed at the bottom of the dish. Three replicate dishes were prepared for each of the samples collected and analysed from the extraction. To each of the dishes, 50 µL of the sample was added and then allowed to vaporize for 15 minutes before the introduction of 20 seeds of *P. pratense* to the dish. Along with the seeds, 1 mL of water was also added. The dishes were placed randomly on windowsills, and 3 days of growth were allowed before another 1 mL of water was added to each dish. After the second watering, the dishes were again randomly distributed in the space before they were allowed another 7 days to grow. After a total of 10 days, the number of germinated seeds were counted, and the length

of sprouts was manually measured, using a ruler, and data was compiled. For the allelopathy study of the pure compounds, 6 solutions were prepared, in accordance with the calculated amount based on the average signal intensity of all extraction samples. The signal intensity of the analyte peak was normalized to that of the internal standard peak for each analysis. One solution of α-pinene with a concentration of 50.05 µg/mL in hexane, and one of caryophyllene with a concentration of 9.86 µg/mL in hexane were prepared in accordance with the results obtained from the analyses. One blend of the two terpenes was prepared in hexane with the same concentrations as for the individual solutions. Finally, 3 solutions were prepared with concentrations 100-fold higher than the previously described. Hexane blanks were also prepared to be used as controls in the allelopathic study of the pure compounds.

Hazards

During the time in the laboratory at KTH, the students and the personnel wore protective gear (lab coats, gloves, protective goggles). All SPE samples were prepared in a fume hood, and solvents were prepared by the supervisor. All dispensing of solvent volumes larger than 5 mL was performed by the supervisor. The students were given information regarding the hazards of the solvents and what precautions that were needed in order to handle the samples. Sample aliquots were kept in separate 1 mL vials so that the students did not have to handle larger volumes of solvent at once during allelopathic trials. Furthermore, samples were stored in PP tubes sealed with lids and parafilm in order to minimize the amount of sample that could evaporate during handling and transport. Samples were kept in freezers at both locations during storage. Disposal of samples were done in accordance with guidelines from KTH and SEKA Miljöteknik AB, and was performed at KTH.

RESULTS AND DISCUSSION

Part I: Extraction and Gas Chromatography – Mass Spectrometry analysis of extracts

In figure 2, the comparison of the samples obtained from the SPE first elution step from the second replicate of both the 0.5 g/mL extractions and the 1 g/mL extractions collected during the three rounds are shown. In the beginning of the project, the students were asked to perform the extractions following an already existing protocol for UAE together with a SPE concentration step (Supporting Information S4). The students were tasked with preparing two different sets of extractions (with different plant mass-to-solvent ratios) for each of the three rounds of sample preparation. This was done so that the students could observe any differences in the peak areas of the compounds when increasing the parameter of mass-to-solvent ratio. After the extraction and concentration steps were performed, the internal standard was added so that the

analyte peak areas could be normalized to the peak area of the internal standard. Thus, the results between rounds could be compared.

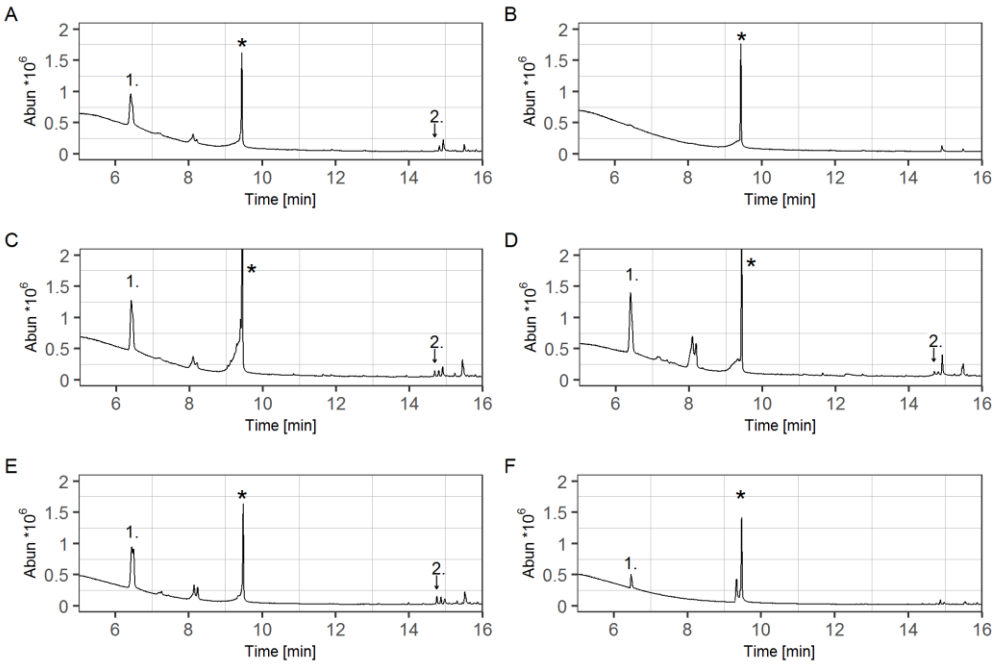


Figure 22: A – 0.5 g/mL elution I from round 1, B – 1 g/mL elution I from round 1, C – 0.5 g/mL elution I from round 2, D – 1 g/mL elution I from round 2, E – 0.5 g/mL elution I from round 3, F – 1 g/mL elution I from round 3. All shown chromatograms are the analysis of replicate 2 from respective round of each mass-to-solvent ratio. Heptyl acetate was used as an internal standard and its peak is labelled with an (*). Labels 1 and 2 show the peaks for α-pinene and caryophyllene respectively if present in sample.

After the extraction samples had been analyzed, the students were given tools for peak detection through the peak detection method described in the Procedures section. An aid in the form of a programmed Excel file for calculating the retention index of the peaks[20] using a C_7-C_{40} alkane standard was also provided. Moreover, to strengthen the identification, external standards were analyzed with the same GC-MS method as the extraction samples. Using these three tools, two compounds were identified with all three methods, namely the monoterpene α-pinene, identified in other studies of Ground Elder[21-24], and the

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sesquiterpene β-caryophyllene, also identified in other studies of the plant[21,22,24]. All data handling was then performed with these compounds in mind, as no other compound was identified with all three identification methods. This also helped narrow down the blend of compounds that the students would perform allelopathic trials with later in the project. The normalized average area data for the peaks of the terpenes in the respective sample are shown in table 1. The normalized area is calculated as the quotient of the area of the terpene and the area of the internal standard. Here, it can be seen that there is a large variation in the areas obtained from the repeated analysis of the two studied compounds. In figure 3, the peak areas for the two selected target compounds normalized using the peak area of the internal standard are shown as boxplots, to visualize the mean and standard deviation calculated from each of the rounds. The calculations for these statistical metrics were performed by the supervisor as the students focused on the statistical analysis of the allelopathic data presented in part II. It was determined that the students would perform the statistical evaluation on their growth data due to the larger number of datapoints. The larger number of datapoints would make trends and correlations of the data clearer for the students to see, thus facilitating the understanding of the results. In correspondence to table 1, it can be seen where the outliers, marked by the dots in figure3A-B, can be found. The analyte peak areas were not consistent in size through the replicates and rounds of the two mass-to-solvent ratios used to analyze *A. podagraria*, and caryophyllene was not detected in all samples. Moreover, no significant difference between the two mass-to-solvent ratios could be seen. A bar graph is also shown, comparing the normalized area ratios of the two compounds (figure 3 C). Despite the variations in the results, α-pinene appears as the most abundant compound in all the extractions. Additionally, in figure 3B, it can be seen that no caryophyllene was detected in any of the extractions of the first round, except for one detection of this sesquiterpene in the 0.5 g/mL extraction. Based on the results presented in figure 3, a similarity between round 2 and round 3 could be found with regards to the mean extracted amount of both compounds. Moreover, the ratio between the two terpenes seems to be similar in round 2 and round 3, as seen in figure 3C. This aided in narrowing down the composition of the blend of compounds that the students used to perform allelopathic trials with later in the project.

Table 1: Normalized peak areas (area ratio) of α-pinene and caryophyllene in the two fractions, for replicates R1-R5, and rounds 1-3 for both mass-to-solvent ratios. E 1 – elution I, E 2 – elution II.

	Mass-to-solvent ratio = 0. 5 g/mL									
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 1	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2

α-pinene	0.378	-	0.835	0.030	-	-	1.180	0.023	1.133	0.083
caryophyllene	0.051	-	-	-	-	-	-	-	-	-
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2
α-pinene	0.240	0.037	0.502	0.072	0.124	-	-	0.077	0.251	0.024
caryophyllene	0.063	-	0.024	-	0.219	-	0.077	-	0.065	-
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 3	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2
α-pinene	0.312	0.100	0.496	0.150	0.344	-	0.446	0.409	0.597	0.100
caryophyllene	0.052	-	0.082	-	0.042	-	0.124	-	-	-
Mass-to-solvent ratio = 1 g/mL										
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 1	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2
α-pinene	0.866	0.304	-	-	-	-	0.112	0.070	0.942	0.036
caryophyllene	-	-	-	-	-	-	-	-	-	-
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2
α-pinene	0.491	0.193	0.913	0.025	1.164	0.032	0.599	0.196	0.417	0.140
caryophyllene	0.034	-	0.061	-	0.086	0.034	0.054	0.029	0.083	-
	R1 Area quoteratio		*R2 Area quoteratio*		*R3 Area quoteratio*		*R4 Area quoteratio*		*R5 Area quoteratio*	
Round 3	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2	E 1	E 2
α-pinene	0.815	0.465	0.101	0.194	0.043	0.053	-	0.191	0.544	0.240
caryophyllene	0.063	0.050	-	-	-	-	-	-	-	0.082

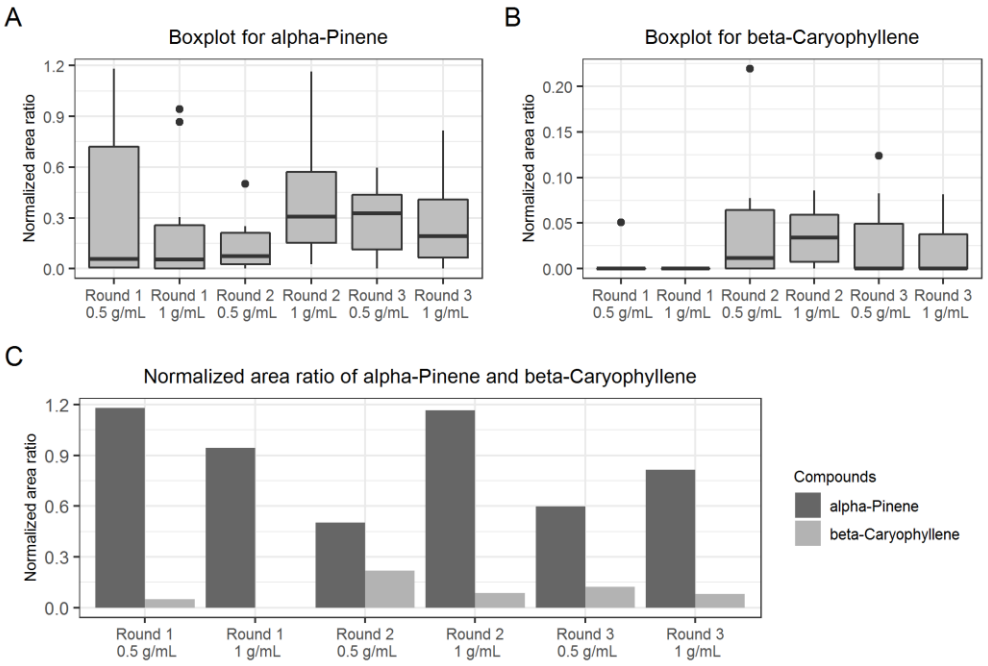


Figure 3: A - Boxplot describing the mean and standard deviation of the peak area ratio of alpha-pinene and internal standard in all 6 sample rounds, B - Boxplot describing the mean and standard deviation of the peak area ratio of beta-caryophyllene and internal standard in all 6 sample rounds, C – bar graph comparing the mean area ratio of alpha-pinene and beta-caryophyllene respectively and internal standard for all 6 sample rounds.

Part II: Allelopathic study of the effect of the extracts on *Phleum pratense*

The extracts prepared for GC-MS analysis were after analysis used for allelopathic studies on the *P. pratense* seeds. This is in agreement with the procedures for the allelopathy trials that have been performed by other students at BGYA prior to this study. When the sprouts had been counted and measured, the datasets obtained from the growth studies were used to calculate the mean, standard deviation, and variance of the parameters in the datasets**.** This was done in a similar way as for the peak area of the two target terpenes and was performed by the students. In figure 4, the histograms show the frequency distribution of sprout length between the two different mass-to-solvent ratios, and the two different elutions. Also shown in figure 4 are the means of the allelopathic trials, marked by the dashed lines, and the means of the corresponding control samples, marked by the solid line. The means of the sprout length were calculated based on the data from all three rounds of the extractions, including the seeds that did not sprout (0 cm). For each round of extraction, a total of 600 seeds were set to evaluate the effect of the extracts on *P. pratense*, 300 for each of the two elutions. This gave 900 measurements in total

for all three rounds of extractions for each of the solvent-to-mass ratios, resulting in a total of 1800 measurements for the entire study. In total, 1 146 seeds sprung sprouts for the 0.5 g/mL ratio extracts, and 1 140 seeds sprung sprouts for the 1 g/mL extracts. Thus, no significant differences were found between the two mass-to-volume ratios in this part, either. The seeds that did not spring any sprouts (Table 2), and thus were given a length of 0, were omitted from the figure to allow for a clearer overview of the frequency distribution of the recorded lengths. In figure 4A-D there are accumulations of visibly higher counts in the whole numbers 1, 1.5, 2 and 2.5 cm, which most probably arises from the manual measuring method of the sprout length. This suggests that the manual method of measuring influences the normality of the data, which was investigated further using the statistical metric of Shapiro-Wilk's test. In figure 4, the values shown are obtained from testing the datasets for normality using Shapiro-Wilk's test, where the p-values are reported based on a 95% confidence level. The data from the growth trial do not appear to follow the normal distribution, as supported by the Shapiro-Wilk's test, as no p-values were reported to be >0.05. This calls for caution when calculating the statistical properties, as certain metrics are calculated differently depending on the normality of the data. However, such adaptations are outside of the scope for the academic level of the high-schools students performing this study. If a similar project were to be given to students in a higher level of education, where the understanding and application of statistics are more developed, the statistical study could be broadened.

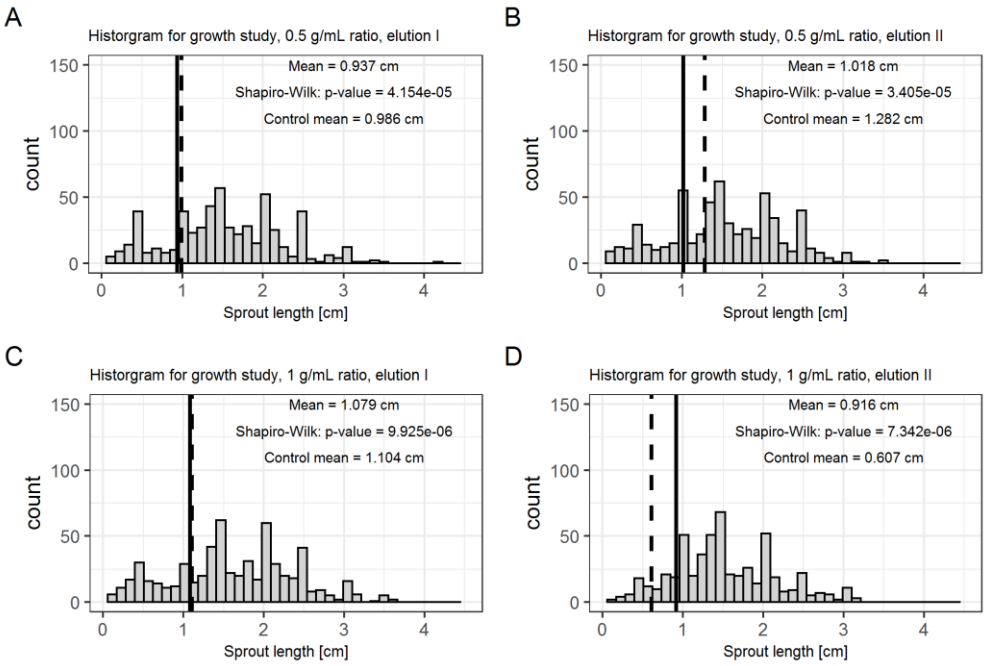


Figure 4: A - Growth histograms for Elution I with a 0.5 g/mL ratio, marked with mean, mean of control and metric of normality, B - Growth histograms for Elution II with a 0.5 g/mL ratio, marked with mean(solid line), mean of control (dashed line) and metric of normality, C- Growth histograms for Elution I with a 1g/mL ratio, marked with mean, mean of control and metric of normality, D - Growth histograms for Elution II with a 1 g/mL ratio, marked with mean, mean of control and metric of normality

Based on the number of seeds that did not sprout, table 2 shows the degree of seed germination inhibition for each of the elutions, and their mass-to-solvent ratio. In table 2, it can be seen that there is no clear pattern between the two different mass-to-solvent rations nor the elutions. The average percentage of inhibition between the four reported categories is 36.5 %, with a rough variation of 3 percent. Furthermore, the control sample for elution II in the 1 g/mL extraction is the only control which showed a higher percentage of inhibition than its corresponding extract trial. It was postulated that the extracts of Elder ground might contain too low concentrations of the allelochemicals in order to obtain a definite difference between the extracts and the blanks. Therefore, the investigation of the identified terpenes' effect on *P. pratense* was performed.

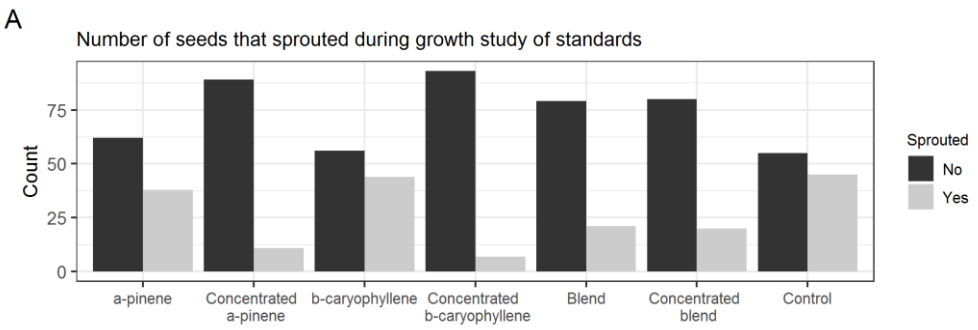
Table 2: Description of seeds germination inhibition by the different elutions of the two mass-to-solvent ratios. The inhibition percentages of the control samples are also shown, as well as the differences between the extract samples and their corresponding control.

Sample	***Number of seeds***	***Number of non-sprouted seeds***	***Inhibition [%]***	***Difference [%]***

Elution I, 0.5 g/mL	900	350	38.89	
				7.78
Control Elution I, 0.5 g/mL	180	56	31.11	
Elution II, 0.5 g/mL	900	304	33.78	
				8.78
Control Elution II, 0.5 g/mL	180	45	25.00	
Elution I, 1 g/mL	900	303	33.67	
				9.78
Control Elution I, 1 g/mL	180	43	23.89	
Elution II, 1 g/mL	900	357	39.67	
				18.67
Control Elution II, 1 g/mL	180	105	58.33	

Part III: Allelopathic study of synthetic blends of α-pinene and caryophyllene on *Phleum pretense*

The third part of the project concerned testing the allelopathic effect of the two specific terpenes identified in part I of the project as pure compounds. This was performed as to investigate if they contributed to the allelopathic effect postulated in part II. Due to restricted time allowed in the lab because of the COVID-19 pandemic, it was determined that an average between the two concentrations from the different mass-to-solvent extraction ratios would be tried. The concentrations were calculated by multiplying the signal quotient between the analyte peak and the internal standard peak, with the concentration of internal standard, that was known from the sample preparation. Thus, three solutions were prepared: one with the selected concentration of α-pinene in hexane, one with the selected concentration of caryophyllene in hexane and one solution with the two compounds blended in hexane. In addition, three solutions with the same composition but 100-fold concentration were prepared. The concentrated samples were prepared so that the students could see the effects of the terpenes on the seeds, if the observed amounts from the extractions were too low to elicit an effect. Ideally, two blends should be prepared to represent the two different extraction ratios, but as the time in lab was restricted, an average blend was used so that the students may obtain results to complete the work. The two compounds were tested for their allelopathic effect on *P. pratense*, both individually and in mixtures, as to investigate if there was any joint effect of the compounds in their allelopathic abilities, or if the compounds were more potent when they were used individually.



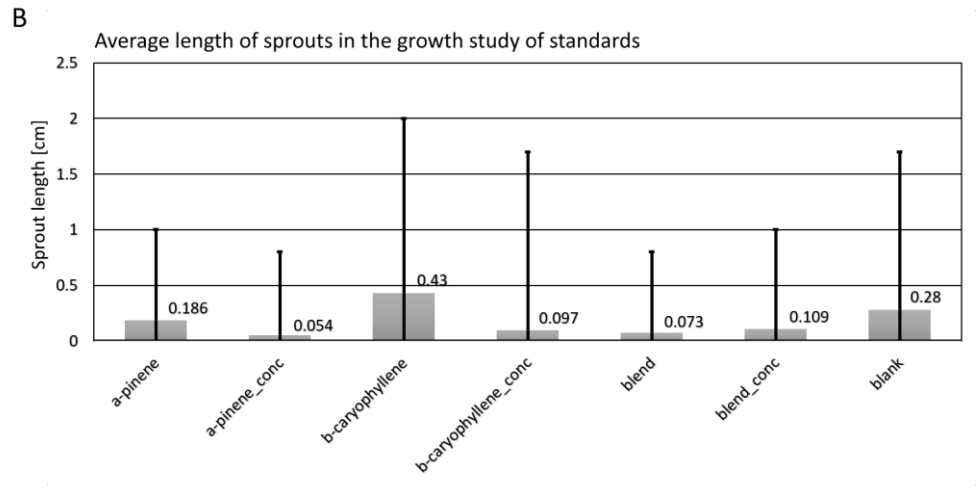


Figure 5: A-Number of seeds that shot sprouts during the growth study of the pure compounds, B - The average length of sprouts that shot sprouts during the growth study of the pure compounds (bars show min and max lengths).

Similarly, to the growth study of the plant extracts, 5 replicates of 20 seeds were used to evaluate the allelopathic effect of the individual compounds and the blend. Using the compounds separately in the concentrations determined from the GC-MS analysis, there was a similar distribution between the number of seeds that shot sprouts and those that did not, compared to the control. The control in this case was GC-grade hexane, which was also used to dissolve the pure compounds when preparing the solutions. This suggests that the inhibition of sprouting might be due to the use of hexane, rather than the use of the terpene. On the other hand, it can be seen that the concentrated solutions of the isolated terpenes show a considerable reduction in the number of seeds that sprouted. Out of the total 100 seeds used, only 7 and 11 seeds sprouted

in the concentrated solutions of α-pinene and caryophyllene, respectively. This effect is not as pronounced in the germination results from the blend samples, where roughly 20 of the seeds sprouted in the trials for the blend representative to the amounts detected in the extracts, as well as for the more concentrated blend. It appears as if increasing the concentration for the blend does little to further inhibit germination of seeds, unlike when the terpenes are used separately. Figure 5B represents the average length of the sprouts (including seeds that did not sprout as 0 cm), which shows that the average length of the sprouts that grew in the hexane control was 0.28 cm. Comparing this result to that of the length for the solution of the lower concentration of β-caryophyllene, it can be seen that the sprouts grew longer in the latter samples. In conjunction with the number of seeds that were germinated, when compared to the control, it can be argued that there seems to be no allelopathic effect of caryophyllene on the timothy seeds. However, α-pinene showed some allelopathic properties, as the average length of the sprouts were shorter than that of the control, in addition to the slightly larger portion of seeds not shooting any sprouts. The concentrated solutions showed a significant decrease in average sprout length, which was in line with an overall allelopathic effect of the increased concentration in relation to what was detected in the plant extractions. Finally, much like the number of seeds that sprouted, there is a decrease in average length of the sprouts when comparing the blends with the control, but not a large difference when comparing the blend and the concentrated blend solution. It could be concluded that there was a stronger allelopathic effect from α-pinene than from caryophyllene with regards to the number of seeds that were germinated, and average length of the sprouts. It could also be determined that there is a concentration-dependent relationship on the extent of allelopathic effect for both of the isolated compounds. Finally, it could be concluded that there is a joint allelopathic effect when using the two terpenes in a blend representative to the amounts detected in the extracts of *A. podagraria*. An effect that does not seem to be increased with increased concentration.

Student evaluation and development of the project

After performing the laboratory work and finalizing their report, the students were asked to fill out a small survey in order to gain insight to their thoughts of the project. This was the first time the project was performed with this structure, that is, with a larger allelopathic study and the testing of individual compounds found in the extracts. The feedback from students was limited due to the low number of participants, but this is still a vital part in the evaluation and further development of this type of high school project work performed in collaboration with a higher education institution as KTH. The survey was anonymous as well as voluntary, and the full answers to the survey questions are available in the Supporting Information, S5. What follows is a brief summary of the responses from the survey along with some thoughts on how to adapt and improve this project work to improve the pedagogical aspects and the experience for future students. To begin with, all students stated that their desired learning outcomes from the project was

to practice scientific methods and apply these to a research topic. Students also stated that they gained insight into what it is like to work with real research topics and the need to be solution oriented. Furthermore, they achieved their desired learning outcome, allowing them to practice the application of a scientific method on a project larger than they usually get the opportunity to do at the high school. The students also agreed on that they were allowed to search for solutions to any problems that occurred along the way of the project. At the same time, they felt comfortable in seeking help from their supervisors if they felt they needed aid in finding said solution. However, a consensus can be discerned from the survey concerning that more explanations were required regarding certain parts of the project, and that some aspects were more difficult to obtain information about and understanding by themselves. This feeds into the students' advice to any other high school students who would perform a similar study in the future, that more enjoyment and understanding can be gained if one does more reading prior to the study. In general, the project was suitable for high school students, but in order to meet the feedback from the students, implementation of guided literature search sessions could be a viable modification of the project. Such a session was implemented in the beginning of this project to give the students a more solid foundation of knowledge to lean on when starting the laboratory work. A second literature session was also implemented after the laboratory part has been finished, and the writing of the final report begins. However, auxiliary sessions could be scheduled as to provide more information and chances to ask questions, as to improve this point in accordance to the feedback of the students. This could help guide the students to find the appropriate reading material in order to answer queries regarding methods and results that have emerged during the course of the project. It could also be advisable for supervisors to set the correct frame of mind for their students regarding the extent of understanding required from them in order to reach a passing grade in the project. The understanding and adaptation of a GC-MS method is outside the scope of knowledge that is demanded from a Swedish high school student. Their understanding of the analytical instrumentation will increase considerably during the project, but the level at which the students are required to show knowledge and understanding of the method should be moderated by the students' academic level. Here, a clearer syllabus, and planned learning outcomes might have to be described in more detail beforehand, as to show the students the appropriate level of understanding they are required to reach in order to fill the requirement for a pass grade in the project.

ADAPTIONS AND CONCLUSIONS

From this study it was concluded that one important area of improvement for this type of project lies in helping students obtain the appropriate amount of understanding on how they should obtain their data, and why the methods are designed in the way they are. The project presented here could be adapted to a higher academic level, where method development of extraction methods and GC-MS analysis could also be

incorporated. This inclusion of method development would allow students to practice their understanding of the analytical aspects of the project. Additionally, more intricate blends of compounds could be tested in order to further illustrate the ecological effects of the compounds detected in the extracts. This would, however, require more scrutiny of the GC-MS data, and the use of more external standards in order to correctly identify the compounds and abundances. The methodology used is basically applicable to other plants, but the choice of extraction solvent, sample preparation, and GC-MS parameters may have to be adjusted.

The project was appreciated by its conductors and can be modified to also suit bachelor and master students, adapting workload and expected outcomes accordingly.

ASSOCIATED CONTENT

Supporting Information

Supporting Information(docx) including:

S1. Abstracts of high school reports in Diploma projects preceding the one (2021), on which the present work is based

S2. High school report in the Diploma project, on which the present work is based

S3. Photos of *Aegopodium podagraria* (Ground Elder), and *Phleum pretense* (Timothy)

S4. Generic protocol for ultrasound assisted extraction (UAE) and solid phase extraction (SPE)

S5. Student survey

Table 1S. Summary of the number of analyses performed on each sample.

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Supporting Information

Investigation of the chemical inhibition effect of Ground Elder (*Aegopodium podagraria*) on Timothy (*Phleum pratense*) – Introducing high school students to analytical chemistry and chemical ecology

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S1. Abstracts of high school reports in Diploma projects preceding the one (2021), on which the present work is based

High school students at Blackebergs gymnasium, Natural Science Program

2016 Allelopathy in *Aegopodium podagraria*

(originally in Swedish: Allelopati hos *Aegopodium podagraria*)

Students: Edit Jonsson, Michael Mekonnen and Susanna Olsson
Supervisors: Prof. Anna-Karin Borg-Karlson and Assoc. Prof. Raimondas Mozuraitis, Organic Chemistry, KTH, and Annika Norin, PhD and Leena Arvanitis, PhD, Blackebergs Gymnasium

Aegopodium podagraria is an invasive weed known for its ability to take over areas where other plants are already living. Some plants species secrete allelochemicals in order to affect the growth of other plants in their surroundings. Our hypothesis was that *A. podagraria* uses allelochemicals to inhibit the growth of their competitors such as *Phleum pratense*. We used different extracts of *A. podagraria* to test the effect they had on the germination of *P. pratense* seeds. We found active substances in the extract where we used ethyl acetate as a solvent. The extract was then fractionated and the fractions influence on the germination of *P. pratense* seeds was investigated. Lastly, the fractions were analysed in an attempt to identify the active chemicals.

2017 Allelopathy in *Aegopodium podagraria* and *Cinnamomum burmannii*

(originally in Swedish: Allelopati hos *Aegopodium podagrari* och *Cinnamomum burmannii*)

Students: Johanna Bergsten, Evelina Pajus, Louise Zettergren and Isabella Åström
Supervisors: Prof. Anna-Karin Borg-Karlson, Organic Chemistry, KTH, and Annika Norin, PhD and Leena Arvanitis, PhD, Blackebergs Gymnasium

Some plants have an ability to emit chemicals that decrease the seed germination, the growth, and reproduction ability of surrounding plants in order to facilitate its own growth. The phenomenom is called allelopathy and chemicals with allelopathic effect for allelochemicals. Allelochemicals could be used as herbicides, and in the future replace existing pesticides that contain chemicals that are harmful to the environment. In the present study, seeds from *Lactuca sativa*, *Raphanus sativus* and *Lepidium sativum* were grown in extracts from the potentially allelopathic species *Aegopodium podagraria, Urtica dioica, Saponaria officinalis* and *Cinnamomum burmannii* using distilled water and ethyl acetate as controls. Our results show that extract from *A. podagraria* roots and *C. burmannii* decreased germination of *L. sativa* and *L. sativum* seeds. A one percent solution of *C. burmannii* extract decreased germination of *L. sativum* with 50 % indicating a strong allelopathic effect.

2018 Allelopathy in *Aegopodium podagraria* - The Future of Natural Pesticides

Students: Sara Axelsson, Beata Johansson and Emilia La Corte Lans
Supervisors: Prof. Åsa Emmer, Analytical chemistry KTH, Prof. Anna-Karin Borg-Karlsson, Organic chemistry KTH, and Leena Arvanitis, PhD, and Annika Norin, PhD, Blackebergs gymnasium

Aegopodium podagraria (ground elder) is an invasive species. It is highly successful in out conquering neighboring plants by releasing allelopathic compounds, also known as allelochemicals. Research on its inhibitory effect can benefit the development of natural pesticides. The present study identifies the active substances, how they affect seed germination and growth of *Phleum pratense (Timothy grass).* We used an extract consisting of plant exudates from roots of *A. podagraria* , diluted with ethyl acetate. The extract was fractionated with liquid chromatography. Cultivations of the fractions

were constructed. Those fractions that indicated inhibited growth and seed germination, were further examined. The different fractions were analyzed in gas chromatography and mass-spectrometry, and cross referenced with the KTH (NIST). Our results showed that *A.podagraria* effectively inhibits seed germination and growth in seeds of *Phleum pratense* . We found caryophyllene, myrcene and limonene in fractions that inhibited growth in the culture study, in addition to α-pinene that was found in a previous study. However, to determine if these chemicals are responsible for the allelopathic effect, further research is needed. Future research will be locating more allelochemicals and determining the likelihood of their presence.

2019 Allelopathy in *Aegopodium podagraria*
(originally in Swedish: Allelopati hos *Aegopodium podagraria*)

Students: Filippa Hansson, William Nilsson, Filippa Olsson and Towe Sundvall Malmsten
Supervisors: Prof. Åsa Emmer, Analytical chemistry KTH, and Leena Arvanitis, PhD, and Annika Norin, PhD, Blackebergs gymnasium

Aegopodium podagraria (ground elder) is an invasive species that can be found in most parts of Sweden. *A. podagraria* is highly successful in out conquering neighbouring plants by releasing allelochemicals into the soil. The purpose of our study was to identify some of the allelopathic chemicals present in *A. podagraria.* We used extracts from the roots of *A. podagraria* that were diluted in ethyl acetate. The extracts were refined through a hydrophobic column and later with liquid chromatography. To determine in which fraction the allelopathic chemicals were located we constructed a cultivation with seeds of *Phleum pratense*. The results were analysed with gas chromatography and mass- spectrometry to identify the allelochemicals in *A. podagraria.* Our results showed that *A. podagraria* inhibits growth and seed germination in *P. pratense*. We found Methoxyeugenol and Myristicin in our extracts, as probable allelochemicals in *A. podagraria*. To determine if these chemicals are responsible for the allelopathic effect, further studies would have to be conducted. The identified chemicals can be used to develop natural pesticides in the future.

2020 Allelopathy in *Aegopodium podagraria*
(originally in Swedish: Allelopati hos *Aegopodium podagraria*)

Students: Ishrat Chowdhury, Erik Forsberg and Louise Åkerberg
Supervisors: Prof. Åsa Emmer, Analytical chemistry KTH, and Annika Norin, PhD, and Maria Almlöv, Lic, Blackebergs gymnasium

Aegopodium podagraria (Goutweed) is an invasive plant, widely spread in temperate areas throughout the world, that has proven to perform allelopathy on its neighbouring plants (Hansson *et al.,* 2019). That is, it releases so called allelochemicals which can potentially act as bioherbicides by inhibiting the growth of plants in its surrounding environment. In this study, the allelochemicals released from *A. podagraria* were performed on *Phleum pratense* (Timothy grass). The purpose was to identify the potential allelochemicals in *A*. p*odagraria*. Extraction from the roots of *A. podagraria* from a previous study (Hansson *et al.,* 2019) were diluted in ethylacetate and refined through liquid chromatography. The residual of the substance left in fractions from the previous study were then used to incubate with seeds of *P. pratense*. Fractions where *P. Pratense* had sparse growth indicated allelopathic tendencies. This decided which specific fractions of *A. podagraria* that were further investigated. The assorted fractions were analysed through GC-MS (Gas Chromatography - Mass

Spectrometry), to identify the specific allelochemicals that were responsible for inhibiting the growth of *P. Pratense.* The result of our study presents two different allelochemicals that are potentially active in *A. podagraria.* The discovered allelochemicals are *hexadecanoic methyl ester* and *octadecanoic acid*, which both are fatty acids. Extended knowledge of allelochemicals might be useful in the future development of herbicides. However, further examination of the specific allelochemicals is needed.

Summary of the iterative development of the methodology between different years

Over the years the main idea of studying the allelopathic effect of some plants on others has been maintained. However, different plants (and parts of the plants) have been studied, with the final choice being *Aegopodium podagraria* as the allelopathic plant (roots extracted), and *Phleum pratense* as the plant, which growth was evaluated. In the latest project, the allelopathic investigations was more extensive, and also included studies of two identified extracted compounds.

As for the analytical chemistry methods, the extraction method, the sample preconcentration and clean-up, prefractionation, and GCMS analysis was changed between each year to improve the results. One obstacle in the earlier works, was the low concentrations of the extracted analytes, and the complexity of the samples. This was addressed by improved extraction and sample preparation in the latest project.

S2. High school report in the Diploma project, on which the present work is based

High school students at Blackebergs gymnasium, Natural Science Program

Isolation and Identification of Allelopathic Compounds in *Aegopodium podagraria*

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ABSTRACT

Allelopathy is known as a plant's ability to secrete chemical substances to the environment that contributes harmful or beneficial effects on other plants. A plant with allelopathic attributions often contains several allelochemicals from different chemical groups (Ferguson, Rathinasabapathi., 2003). Combined, they have a more potent inhibitory or stimulatory effect (Kruse *et al.*, 2000). This study aimed to isolate and determinate potential allelopathic compounds in the plant species *Aegopodium podagraria* (ground elder), which is an invasive plant in Sweden (National encyclopedia., 2020), and one of the most common weeds with a vast distribution area (Jonsson *et al.*, 2016). The method used to identify the allelochemicals was Gas-Chromatography Mass-Spectrometry. *A. podagrarias* inhibitory effect was further examined on *Phleum. pratense* through four cultivation studies. The study showed that the allelochemicals α-pinene and β-caryophyllene are present in *A. podagraria* and have an inhibitory effect on the plant *P. pratenses* germination. Moreover, the study showed that the concentration of the allelochemicals is significant for allelopathic efficacy when α-pinene and β-caryophyllene are not blended. Though, when α-pinene and β-caryophyllene are combined, the concentration of the chemicals is not substantial, and they have a synergistic effect on *P. pratense*.

KEYWORDS Allelopathy, cultivation, *Aegopodium podagraria*.

Introduction

Allelopathy is known as a plant's ability to secrete chemical substances to the environment that contributes harmful or beneficial effects on other plants. The word allelopathy originates from the Greek-derived compounds *allelo,* meaning "of each other," and *pathos* meaning "to suffer.*"* Thus, the term can be translated to "harmful to the other" (Willis., 2007). Allelochemicals exist in several places in the plant, for instance, in the roots, the leaves, and the pollen. During a growing season, the secretion of allelopathic chemicals varies (Ferguson, Rathinasabapathi., 2003) and secrets differently depending on where it is in the plant, for instance, through secretion, evaporation, or putrefactive. Allelochemicals secret not only from plants but from microorganisms as well. The produced allelopathic chemicals are not necessary for the plant´s primary metabolism; therefore, allelochemicals are classified as secondary metabolites (Gross., 2009). The concentration of allelopathic chemicals differs in the plant

tissue and over time. However, the distribution of these chemicals in different plants is, in most cases, unknown (Singh Rana., 2018).

A plant with allelopathic attributions often contains several allelochemicals from different chemical groups such as amino acids, phenolic compounds, carbohydrates, alkaloids, and steroids (Ferguson, Rathinasabapathi., 2003). Combined, they have a more potent inhibitory or stimulatory effect (Kruse *et al.,* 2000). For instance, the sesquiterpene β-caryophyllene (Figure 2) has a potential allelopathic effect (Wang *et al.,* 2009), as well as the terpene α-pinene (Figure 1) has an inhibitory effect (Olenici *et al.,* 2007). Allelopathic weed suppression is affected by physiological and environmental stresses, solar power, lack of appropriate temperature levels, and optimal nutrient. Furthermore, this means that the secretion of allelochemicals depends on the plant's environment (Kruse *et al.,* 2000).

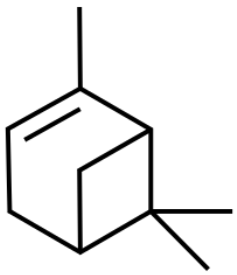


Figure 1: Structural formula of α-pinene.

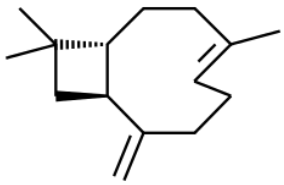


Figure 2: Structural formula of β-caryophyllene.

The function of the secretion of allelochemicals is to counteract against different stress factors from the environment. Exposition of allelochemicals to susceptible plants may lead to the affection of the plant's germination, development, and growth. The most frequently reported effects on plants are inhibited or retarded seed germination, effects on growth, and root development (Kruse *et al.,* 2000). The allelochemicals can also have an inhibitory effect on the growth of coming seasons, thus evaporation in the rhizosphere (Olsson., 2015). This means that allelopathic plants can have an evolutionary advantage (Hallberg., 2017).

The plant species *Aegopodium podagraria* (ground elder) originates from the Middle East (National encyclopedia, 2020), is an invasive plant and is fast spreading in chiefly newly established environments. It blossoms during June – July. The branched root system enables it to grow densely (Anderberg., 2005). The plant is one of the most common weeds in Sweden and has a great distribution area (Jonsson *et al.,* 2016).

Solid-Phase Extraction (SPE) is used to purify and concentrate samples. SPE contains three steps: retention, rinsing, and elution. Substances will either retain, be washed off, or eluted through each step (Simpson., 2000). Gas Chromatography (GC) is used to analyze gas and volatile compounds. In the GC is a mixture of substances gasified and carried by a stream of inert gas passed through a stationary liquid phase in a long column. The substances can be detected and registered with a detector when the substances have passed through the column (National Encyclopedia., 2021). After that, the substances reach Mass Spectroscopy (MS), a method for determining the masses of individual positive or negative ions with a mass spectrometer. Positively ionized atoms or molecules are accelerated in a high vacuum by a strong and homogeneous electric field. A narrow bundle of ions must first pass an electric and then a magnetic field with the lines of force perpendicular to the direction of the beam. The ions are deflected, less the heavier they are, and then hit the detector, an electron multiplier, which measures the ion current. Ions with the same ratio between mass and charge hit the

sensor simultaneously. If ions with different masses are present, the detector registers a mass spectrum with a signal for each ion type with an intensity proportional to the relative presence of the ion type (National Encyclopedia., 2021).

Moreover, this study could contribute to new knowledge about specific plant allelopathy. This knowledge about specific plant allelopathy is used to develop non-pollution pesticides. In addition, it is possible to use blends of allelochemicals in plant extracts to effectively combat specific weeds (Farooq *et al.*, 2011). It could be possible to specify these pesticides on weeds and thus not affect other organisms. The use of allelopathic chemicals in pesticides may also reduce the risk of pesticide resistance on weed.

This study aims to facilitate future research in this field to provide a helpful reference for specific plant allelopathy. Therefore, this study aims to isolate and determine eventual allelopathic compounds in *A. podagraria.*

Materials and Methods

The *A. podagraria* roots and stem were retrieved (2020-09-02) from Råcksta träsk (59°21'05.0"N 17°52'41.2"E). It was washed and kept in a freezer (-84 °C).

Sample preparation

One set of 5 replicates for two different plant mass-to-solvent ratios were prepared for ultrasound extraction on three separate occasions, along with one control sample with only the used solvent. For the extractions, only roots were used, and the two ratios used in the sample preparation were 0.5g/mL and 1 g/mL. After the roots had been placed in a falcon tube and weighed, the appropriate volume of LC-grade acetonitrile (MerckMillipore, Burlington, MA, USA) to achieve the right concentration was added to the falcon tube. The samples were let to soak for 30 minutes before sonification, after which they were placed in a Sonorex RK100H (Bandelin, Berlin, Germany) ultrasonic bath for 60 minutes. The extracts were then removed and filtered through a 0.2 µm polypropylene filter.

The extracts were then purified and concentrated using Bond Elut (Agilent Technologies, Santa Clara, CA, USA) Solid Phase Extraction cartridges. The cartridges have a C18 stationary phase, a particle size of 40 µm, 200 mg bed mass, and 3 mL cartridge volume. For each sample, a cartridge was washed with 3 mL of a 1:3 volumetric ratio solution of Gas-Chromatography-grade ethyl acetate (Sigma Aldrich, St. Louis, MO, USA) and GC-grade hexane (Sigma Aldrich) then conditioned with 3 mL of LC-grade acetonitrile (Sigma Aldrich). The sample was then loaded onto the cartridge bed, after which the bed was washed with 1 mL of a 1:8 volumetric ratio solution of GC-grade ethyl acetate and LC-grade acetonitrile and then eluted with 1 mL of a 1:3 volumetric ratio solution of GC-grade ethyl acetate and GC-grade hexane. Both 1 mL fractions were collected for Gas-Chromatography and Mass-Spectrometry (GC-MS) analysis and cultivation studies. Before GC-MS analysis, 6 µL of an 8.62 mg/mL solution of heptyl acetate (Sigma Aldrich) in hexane was added to 594 µL of each sample as internal standard, resulting in 86.2 µg of internal standard in every sample.

Gas-Chromatography Mass Spectrometry

A 7890A GC system (Agilent Technologies), equipped with a 30 m x 0.25 mm x 0.25 µm DB-5MS column (Agilent Technologies), coupled to a 5975C mass spectrometric detector (Agilent Technologies) was used for all analysis. The GC oven was programmed to execute a 28-minute-long temperature program starting at 40 °C and maintaining this temperature for 1 minute, followed by a temperature gradient of 10 °C/min until a temperature of 260 ° C was reached which then held for 5 minutes. A Cooled Injection System (CIS) (Gerstel, Mülheim an

der Ruhr, Germany) was used as an injection port. It was programmed to execute a temperature program starting at an initial temperature of 40 °C for 6 seconds, followed by a temperature gradient of 12 °C/s until the injector port reached 260 °C to hold this temperature for 2 minutes finally. The ion source temperature was set to 230 °C, the mass range was set to 35-425 m/z, electron ionization was 70 eV, and a solvent vent was set to the first 4 minutes of each analysis. Helium was used as a carrier gas, and a MulitPurpose Sampler (MPS) (Gerstel) was used to inject 1 µL of sample per injection.

Handling of GC-MS data

Peak detection was performed using the integration function in Chemstations (Agilent Technologies) data analysis software with the integration events set as follows; Initial area rejects set to 1500000, initial peak width was set to 0.02, shoulder detection was set to off, and the initial threshold was set to 15.0. Identification with mass spectrometry was made by comparison of the recorded mass spectrum to that of the NIST NIST/EPA/NIH Mass Spectral Library version 2.0 g, retention indices were calculated by comparison of retention times to that of the 49452-U C7-C40 alkane standard (Supelco, Bellefonte, PA, USA) (10 µg of each in hexane) and external standard identification was made by comparing sample analyte peaks retention times to that of the retention times of terpenes present in the CRM40755 Cannabis Terpene Mix A (Sigma Aldrich)(10 µg of each in hexane). During the analysis of the samples, it was randomly selected which samples were to be analyzed in triplicate to show the stability of the GC-MS method. The dispersion of sample runs is shown in the supporting information.

Cultivation studies with root excrete

The cultivations were grown on filter papers in Petri dishes. In the cultivation studies, every fraction (elute and wash) of the five replicates of the plant mass-to-solvent ratios (0,5g/mL and 1g/mL) was used to prepare three Petri dishes (three replicates of each fraction) with 50 µL. The control sample fractions (elute and wash) of the mass-to-solvent-ratios (0,5g/mL and 1g/mL) were also used for the preparation of three Petri dishes with 50 µL each. In the preparation of the filter papers, they were let to soak for 15 minutes before 20 seeds of *Phleum pratense* (Timothy) were placed in each Petri dish. The Petri dishes were then watered with 1 mL of water and enclosed.

Three separate cultivation studies were performed. Since two replicates of the control sample were used in the first cultivation, it consisted of 84 Petri dishes. In the second and third studies, only one replicate of the control sample was used. Therefore, the second and third cultivation studies consisted of 72 Petri dishes. The cultivations were let to grow for three whole days, and then they were watered with 1 mL each. After ten days, the total amount of *P. pratense* straws was counted in each petri dish, and the growth of the straws was measured with an accuracy of one decimal. The mean value and standard deviation of germinated seeds and their length were then calculated.

Cultivation studies with pure standards of identified chemicals

The GC-MS survey showed that there was a considerable amount of α-pinene and β-caryophyllene in the root excretions. Therefore, another cultivation study was performed, but the Petri dishes were instead prepared with 50 µL of pure standards of α-pinene, β-caryophyllene, a blend of the two chemicals, or pure hexane. There were two concentrations of the standards. One was concentrated with the same concentrations as they found chemicals in the root excretions, and the other was 100 times as concentrated. The blend had the same concentration as the α-pinene and β-caryophyllene standards. There were five replicates of each standard. Thus, the study with pure standards of the identified chemicals consisted of 35 Petri

dishes. After the preparation of the filter papers, they were let to soak for 15 minutes before 20 seeds of *P. pratense* were placed on each petri dish. The Petri dishes were then prepared with 1 mL of water and enclosed. The cultivations were let to grow for three whole days before 1 mL of water was prepared on each petri dish. After ten days, the total amount of *P. pratense* straws was counted in each Petri dish, and the growth of the straws was measured with an accuracy of one decimal. The mean value and standard deviation of germinated seeds and their length were then calculated.

Results

In the GC-MS survey was significant amounts of α-pinene and β-caryophyllene detected. Figure 3 shows that α-pinene was detected in every examined wash fraction except the 1 g/mL wash fraction in study 1. However, there was no indication that β-caryophyllene was present in the wash fractions. β-caryophyllene was instead found in the 0,5 g/mL elute and 1 g/mL elute fraction in study 2 and 0,5 g/mL elute fraction in study 3 (figure 4). Moreover, all the samples that contained β-caryophyllene also contained α-pinene.

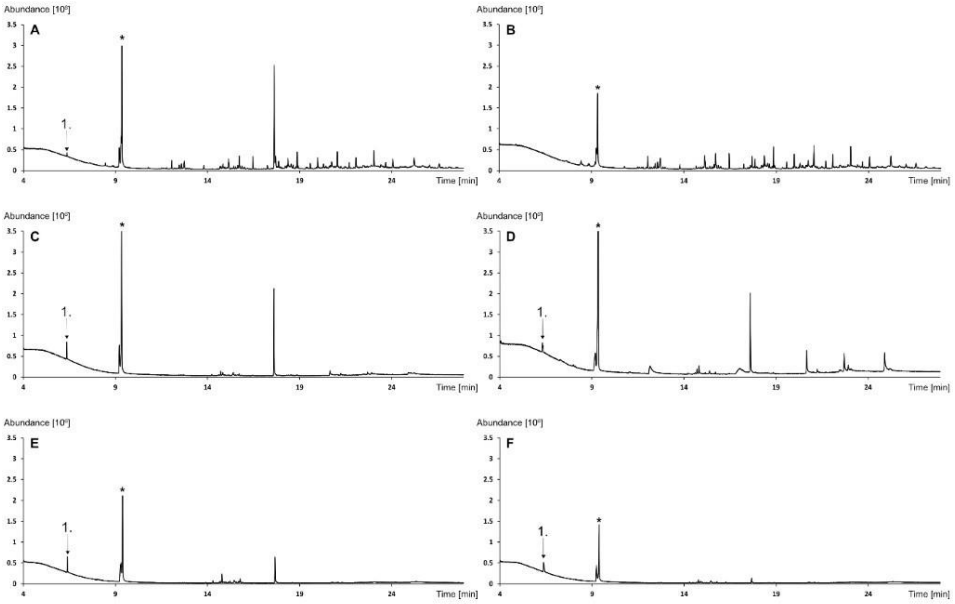
In cultivation study 1, the average germinated *P. pratense* seeds were relatively alike irrespective of prepared fraction (figure 5). However, all the fractions except the elute 1 g/mL had a lower average of germination than the corresponding control sample. On the other hand, the seeds with elute 1 g/mL had the highest variation of germinated seeds and, therefore, the highest standard deviation. The seeds prepared with control 1g/mL wash and control 1g/mL elute had the lowest average length in the study (figure 6). Furthermore, the wash 0,5 g/mL and control wash 0,5 g/mL had a very similar average length, as well as the elute 0,5 g/mL and control 0,5 g/mL elute. Nevertheless, the seeds prepared with the wash 0,5 g/mL and 0,5 g/mL elute were somewhat lower than the appurtenant control sample. The standard deviation of *P. pratenses* length was generally larger than the standard deviation of the germinated seeds.

In cultivation study 2, the average number of germinated *P. pratense* was comparably alike (figure 7) in all the Petri dishes. Moreover, all the Petri dishes prepared with root extractions had a lower average of germination than the corresponding control sample, except for the elute 1g/mL. The seeds with the lowest average germination were the seeds prepared with wash 0,5g/mL. Furthermore, the seeds prepared with control 0,5g/mL elute had the highest average germination. The standard deviation of control 0,5g/mL wash, control 0,5g/mL elute, and control 1g/mL were higher than the rest of the fractions. The seeds prepared with wash 0,5g/mL had the highest length compared to control 0,5g/mL (figure 8), and the seeds prepared with control 0,5g/mL had the lowest average length. However, the Petri dishes' average length was relatively similar irrespective of the prepared fraction.

The results of cultivation study 3 were disparate from the other studies. Many Petri dishes had a significant deviation from other Petri dishes prepared with the same sample, and the standard deviation was very high. Therefore, no further results from this study will neither be shown nor used to conclude about *A. podagrarias* allelopathic properties.

In cultivation study 4, the seeds prepared with β-caryophyllene concentrated and α-pinene concentrated had the lowest average of germinated seeds. Furthermore, the seeds prepared with non-concentrated β-caryophyllene and α-pinene had the highest average germinated seeds. These averages were also similar to the control (hexane blank) average. Moreover, the seeds prepared with blend and blend concentrated had an equal average number of germinated seeds. The seeds prepared with blend had the lowest average length. In addition, the mean length of the seeds in non-concentrated α-pinene and α-pinene concentrated were similar. They were

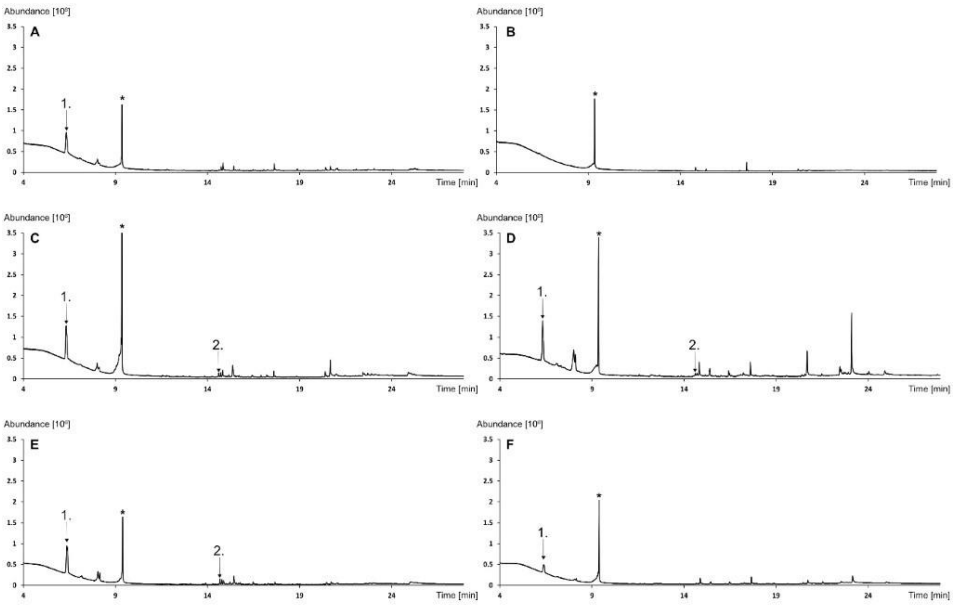
somewhat lower than the average control (hexane blank). However, the control (hexane blank) shows a high standard deviation compared to non-concentrated α-pinene and concentrated α-pinene. The highest mean lengths are shown in the fractions with β-caryophyllene



concentrated, and the seeds prepared with non-concentrated β-caryophyllene had a relatively high average length. Still, the standard deviation is higher in the non-concentrated β-caryophyllene fractions.

Figure 3: **A** – 0.5 g/mL wash fraction from study 1, **B** – 1 g/mL wash fraction from study 1, **C** – 0.5 g/mL wash fraction from study 2, **D** – 1 g/mL wash fraction from study 2, **E** – 0.5 g/mL wash fraction from study 3, **F** – 1 g/mL wash fraction from study 3. All shown chromatograms are the analysis of replicate 2 from respective study of each mass-to-solvent ratio. Heptyl acetate was used as an internal standard and its peak is labelled with an (*). Arrows labelled 1 and 2 show the peak for α-pinene and β-caryophyllene respectively if present in sample.

Figure 4: **A** – 0.5 g/mL elute fraction from study 1, **B** – 1 g/mL elute fraction from study 1, **C** – 0.5 g/mL elute fraction from study 2, **D** – 1 g/mL elute fraction from study 2, **E** – 0.5 g/mL elute fraction from study 3, **F** – 1 g/mL elute fraction from study 3. All shown chromatograms are the analysis of replicate 2 from respective study



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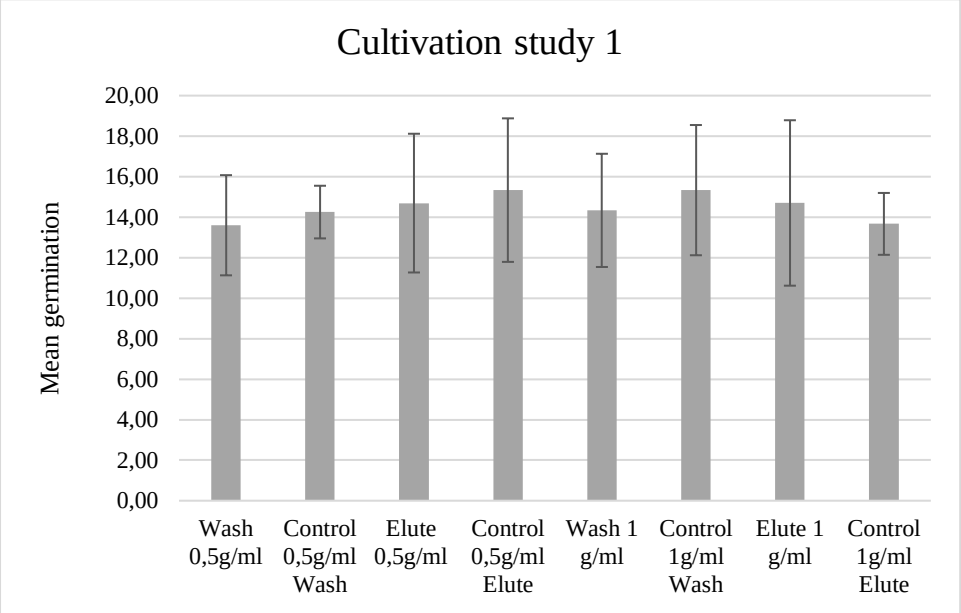

Figure 5: Results from cultivation study 1. Average number of germinated *P. pratense* seeds out of 20 sowed with appurtenant standard deviation.

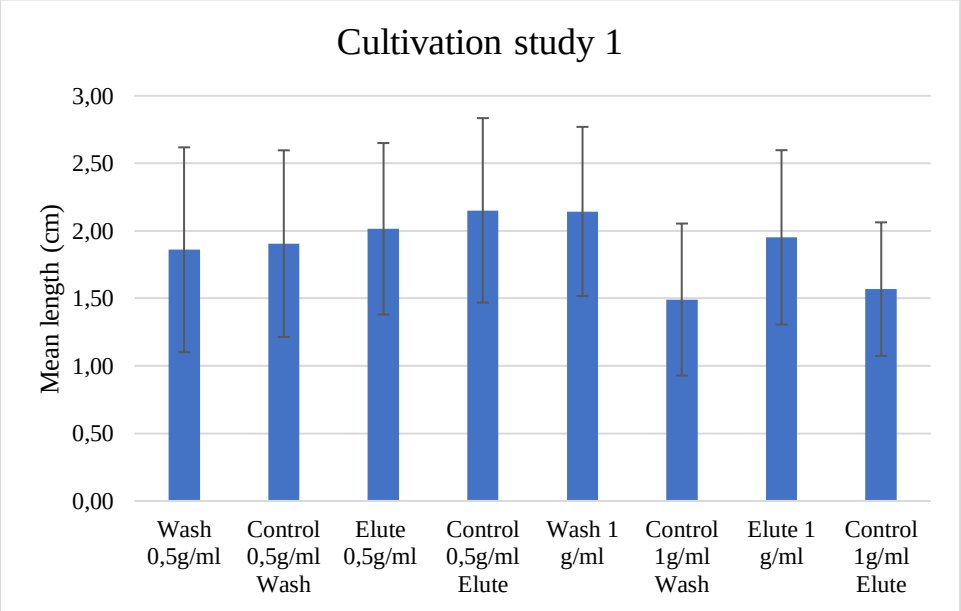

Figure 6: Results from cultivation study 1. Average length (cm) of germinated *P. pratense* with appurtenant standard deviation.

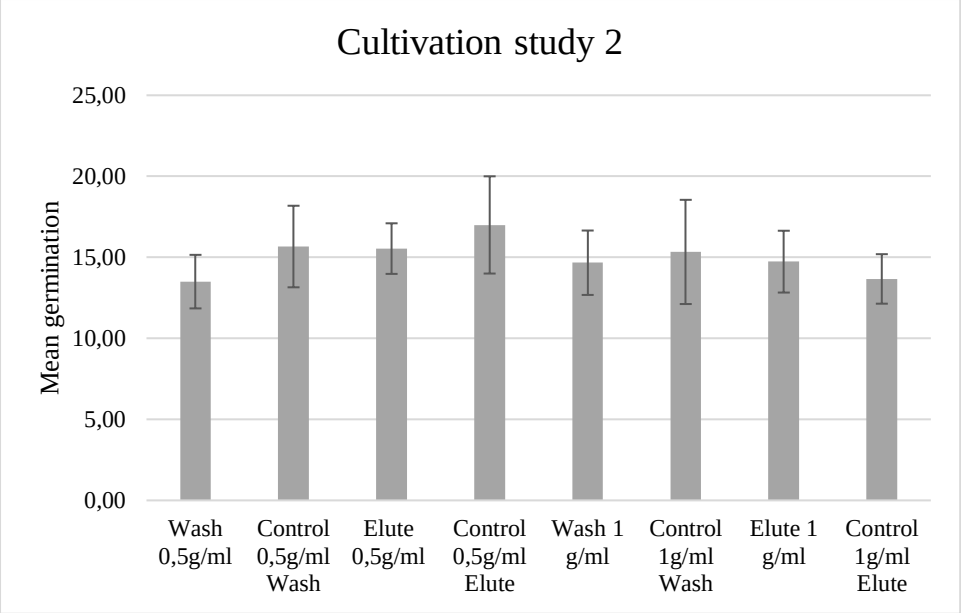

Figure 7: Results from cultivation study 2. Average number of germinated *P. pratense* seeds out of 20 sowed with appurtenant standard deviation.

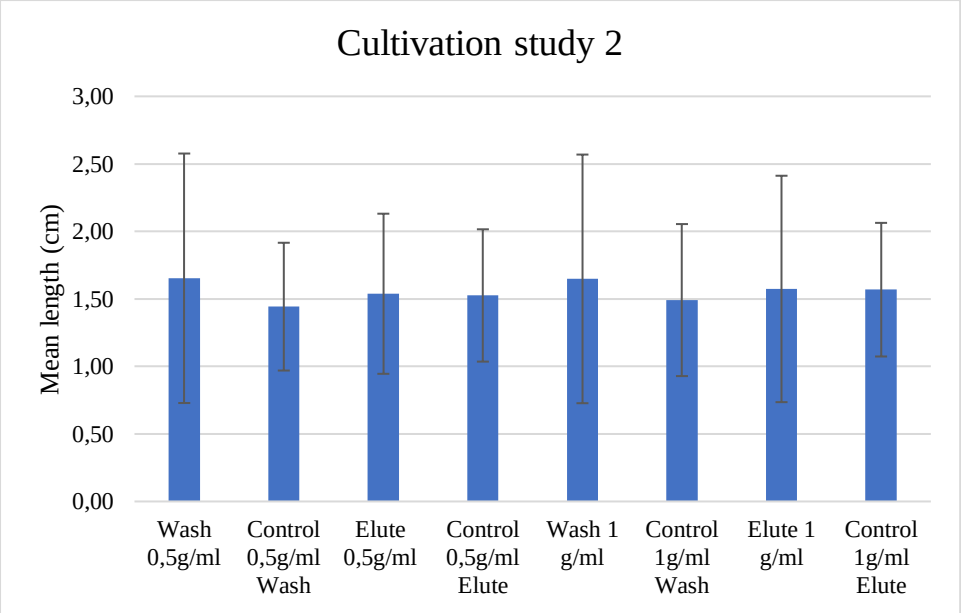

Figure 8: Results from cultivation study 2. Average length (cm) of germinated *P. pratense* in each fraction examined with appurtenant standard deviation.

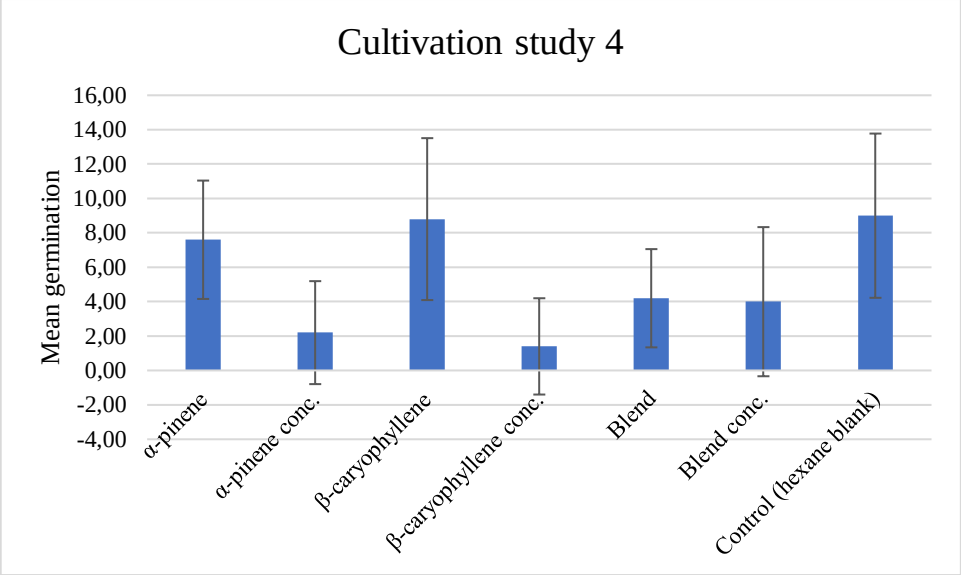

Figure 9: Results from cultivation study 4. Average number of germinated *P. pratense* seeds out of 20 sowed with appurtenant standard deviation. The prepared substances α-pinene and β-caryophyllene are external standards retrieved from KTH. The allelopathic effect of two different concentrations was examined, the concentration found in the root extractions and a concentration 100 times stronger (conc.).

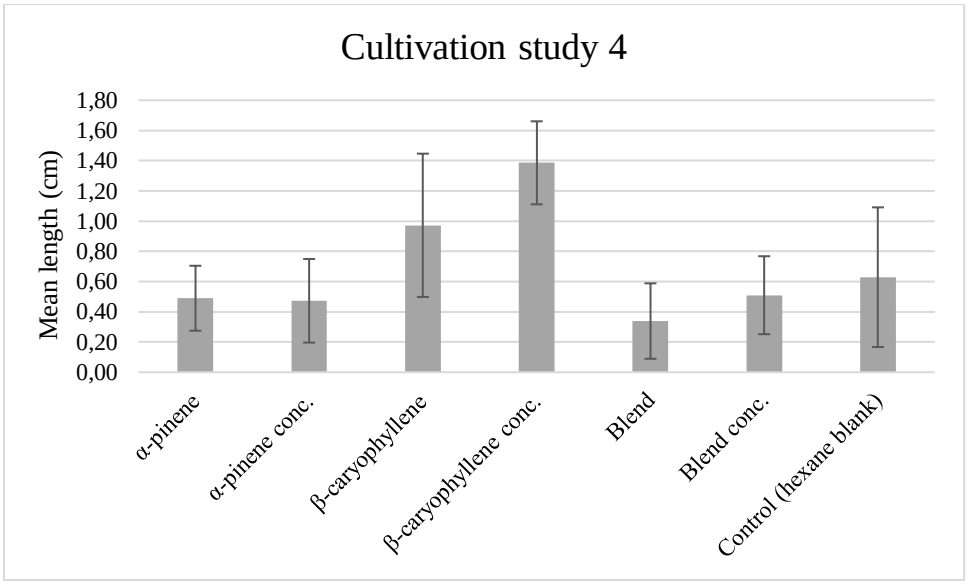

Figure 10: Results from cultivation study 4. Average length (cm) of germinated *P. pratense* in each fraction examined with appurtenant standard deviation. The prepared substances α-pinene and β-caryophyllene are external standards retrieved from KTH. The allelopathic effect of two different concentrations was examined, the concentration found in the root extractions and a concentration 100 times stronger (conc.).

Discussion

The chromatograms (figure 3 and figure 4) show that the terpene α-pinene is present in *A. podagraria*. Furthermore, α-pinene is already an identified allelochemical (Olenici *et al.*, 2007). The chemical is found in every sample examined in the GC-MS except the wash 1 g/mL and elute 1 g/mL fractions in cultivation study 1. β-caryophyllene is also one of the identified allelochemicals in *A. podagraria* (figure 2) and is classified as a sesquiterpene that contains C15-isoprenoids. In previous studies, β-caryophyllene has been identified as a volatile substance that affects other plants (Wang *et al.*, 2009). This allelochemical was found in the

elute 1 g/mL and elute 0,5 g/mL fractions of cultivation study 2. Moreover, the chromatograms show that β-caryophyllene only is found when α-pinene also is present in the elute fractions (figure 4 CD).

The concentrated α-pinene had a fractional higher inhibitory effect on the germination of the seeds than the non-concentrated α-pinene in cultivation study 4 (figure 9). This may indicate that a higher concentration of α-pinene may contribute to a higher inhibitory effect on the plant's germination. However, the seeds prepared with non-concentrated α-pinene and concentrated α-pinene had a relatively similar mean length, which is somewhat lower than the control sample's length (figure 10). Though the difference between the mean length of the Petri dishes prepared with α-pinene and the control is minor, and the standard deviation is significant. Thus, it does not seem that α-pinene affects the plant's ability to grow when germinated regardless of concentration.

Furthermore, the seeds prepared with concentrated β-caryophyllene had the lowest average germination in cultivation study 4 (figure 9). The chemical concentration seems to significantly impact the probability for the seeds to germinate since the seeds prepared with the lower concentration had a high and similar average of germination to the control (hexane blank) in the study. Nevertheless, since it only was one Petri dish prepared with concentrated β-caryophyllene in which seeds had grown, the mean length of the seeds prepared with the fraction can be somewhat deceptive (figure 10). However, even though the data of concentrated β-caryophyllene's effect on the length of *P. pratense is* limited, it is possible to assume that β-caryophyllene does not affect the plant's ability to grow when germinated regardless of concentration.

The seeds prepared with a blend of the two chemicals had a generally low number of germinated seeds, and average length (figure 9 and figure 10) in cultivation study 4, irrespective of the blend was concentrated or not. Thus, the blend of the chemicals in both concentrations seems to inhibit *P. partense's* germination. The seeds prepared with non-concentrated blend had the lowest average length in the study, but it is similar to the control (hexane blank). Moreover, the concentration of the chemicals in a blend does not seem to influence the inhibitory effect on *P. partense's* length nor germination. Since a plant with allelopathic attributions often contains different allelochemicals that have a more potent inhibitory or stimulatory effect combined (Kruse *et al*., 2000), this may indicate that α-pinene and β-caryophyllene in a blend have a synergistic inhibitory effect on *P. pratense.*

In cultivation study 1-3, the germination and length of all the Petri dishes were similar, regardless of the prepared sample contained actual root extraction was a control. Since it only was minor differences between the germination and length in the Petri dishes prepared with root extractions and control sample, it is possible that the root extraction did not have an allelopathic effect in studies 1-3. Furthermore, in cultivation study 1, the seeds prepared with wash 0,5g/mL and wash 1g/mL had the lowest germination and length. According to the GC-MS survey, these samples contained neither α-pinene nor β-caryophyllene (figures 3 and 4). Additionally, if conclusion about α-pinene and β-caryophyllene synergistic effect is correct, the Petri dishes which contained both the chemicals should show a larger inhabitation of germination and length in cultivation study 1-3. Though, the elute 0,5 g/mL and elute 1 g/mL in cultivation study 2, which contained both chemicals, did not show signs to inhibit germination or length (figure 7 and 8). Conceivably, the concentration of the substances was too low to inhibit *P. pratense.* However, there was a considerable variation between the seed's germination and length. This led to that the standard deviation was high, and it was hard to

make substantiated assumptions about the root extractions inhibitory effect on *P. pratense*. This also applies to the length of *P. pratense* in cultivation study 4.

The significant variation and standard deviation between *P. pratense's* germination and length in the Petri dishes may depend on different factors. For instance, the watering was partially non-consistent due to difficulties caused by the Covid-19 pandemic. Likewise, the randomization of the Petri dishes was not repeated. As a result, some Petri dishes were dehydrated. Additionally, the average length of the control (hexane blank) was relatively low in cultivation study 4 (figure 10). Hexane is a volatile substance, indicating that the hexane also dehydrated the Petri dishes. However, these different conditions lead to various opportunities to germinate and grow for the seeds, which could lead to the results shown in cultivation study 1-3 (figure 5-10) and cultivation study 4 (figure 3-4) are somewhat incomparable. Thus, the results of cultivation study 3 are not used to draw any conclusions because too many Petri dishes were dehydrated. Cultivation study 4 was least affected by hydration problems. Therefore, this study is more reliable than the previous three. Furthermore, the whole research was divided into three sets due to lack of time. Still, preferably, the study should have been done in at least seven or more sets to receive more comparable data and reliable results.

In conclusion, this study has identified allelochemicals α-pinene and β-caryophyllene. Other studies show that these have an inhibitory effect on other plants (Wang *et al.*, 2009*; Olenici et al.*, 2007). According to the results of cultivation study 4 (figure 3 and 4), is it possible that α-pinene and β-caryophyllene in a certain concentration have an individual inhibitory effect on *A. podagrarias* germination. However, it does not seem that neither α-pinene nor β-caryophyllene affects the plant's ability to grow after germination, regardless of concentration. Furthermore, when they are blended, the concentration of the chemicals is not substantial for the inhibitory effect on *P. partenses* germination. This may indicate that when α-pinene and β-caryophyllene are combined, they have a synergistic impact on *P. pratense*.

However, this study has only found two potential substances that constitute *A. podagrarias* allelopathic effect. Though a plant with allelopathic attributions often contains several allelochemicals (Ferguson, Rathinasabapathi., 2003), probably, other chemical also affect the efficacy of *A. podagrarias* allelopathy. Furthermore, the secretion of allelochemicals depends on the plant's environment. Thus, factors such as physiological and environmental stress, sun-light exposure, lack of appropriate temperature levels, and optimal nutrient can affect the allelopathic weed suppression (Kruse *et al.*, 2000). Therefore, it would be interesting to compare the allelopathic compounds in *A. podagraria* coming from different environments and during different seasons. Moreover, this study has only examined *A. podagrarias* allelopathic effect on a specific plant, *P. pratense*. Since there are other factors that also impact a plants germination and growth, such as the plant's genetics and environment, the inhibitory effect might be different on other plants. Thus, the cultivation studies should preferably have been done on various plants to receive more data and reliable results. Therefore, further research is necessary to examine *A. podagrarias* allelopathic properties thoroughly.

Since it is possible to use a blend of allelopathic compounds from plant excretions to produce natural pesticides (Farooq *et al*., 2011), this study may provide pesticide companies with helpful knowledge about non-polluting inhibitory substances that can be found in extracts from *A. podagraria.* However, future studies are necessary to examine *A. podagrarias* potential inhibitory effect on other plants more than *P. pratense* to ensure that *A. podagraria* can be used as a part of a natural pesticide.

Acknowledgements

Firstly, we would like to thank Linus Svenberg from the analytical chemistry department, KTH**,** for us a chance to pursue this study and great supervision. We also show deep gratitude to our supervisors at Blackebergs Gymnasium, Annika Norin, PHD and Maria Almlöv, FIL. LIC.

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S3. Photos of *Aegopodium podagraria* (Ground Elder), and *Phleum pretense* (Timothy)

The photos are taken by one of the authors (Å. Emmer), at a later stage. The plant specimens are therefore not the ones used in the high school project.

Aegopodium podagraria

Phleum pretense

S4. Generic protocol for ultrasound assisted extraction (UAE) and solid phase extraction (SPE)

Acetonitrile can be exchanged for other solvents dependent on the plants investigated

UAE

Using the LC-MS grade Acetonitrile, the ratio is 0.1 g/ml of plant mass to extraction solvent.

1. Start by macerating plant material
2. Add extraction solvent
3. Let it soak for 30 minutes in +6 °C
4. Ultrasound for 60 minutes
5. Filter through 0.2 µm PTFE membrane filters

Sample is now ready for SPE concentration

SPE

1. Wash with 3 mL of 1:3 Hexane:Ethyl acetate
2. Condition with 3 mL of Acetonitrile
3. Load sample
4. Wash with 3 mL 1:8 Ethyl acetate:Acetonitrile
5. Elute with 1 mL 1:3 Hexane:Ethyl acetate

S5. Student survey

Student 1

Describe briefly, in your own words, what the project was about? Aim, hypothesis etc
The aim of the study was to isolate and identify possible allelochemicals in Aegopodium Podagraria. Our hypothesis was that it exists allelopathic chemicals in the plant.
Have you done any similar work prior to the project?
No.
How much literature had you read in order to prepare for the project work?
I have read literature on the definition of allelopathy and on the method, for instance GC-MS.
What did you hope to have as a learning outcome from the project?
To learn about the scientific research process.
What did you learn from the project?
Is there anything you though was lacking in regard to learning content? Anything you would have liked more focus on?
I would have liked to have more focus on why we did some steps, for instance why did we use MeCN?
Did you feel that you got sufficient help and support from the supervisors in areas where you felt uncertain? Did you feel that the supervisors where to involved, not letting you discover solutions to problems by yourself?
Yes! No, my supervisor let me discover the solutions to the problems by myself.
What would you say was the most enjoyable part of the project?
To discover the solutions and draw conclusions based on the data.
What was the least enjoyable part of the project?
To wash and dig up the roots.
If this project was to be given to students next year, with the same task as you had, what tips would you give those students in order for them to gain as much as possible from the project?
To read more literature in order to prepare for the project work.
Any other comments?

Student 2

Describe briefly, in your own words, what the project was about? Aim, hypothesis etc
The aim of the study was to determinate and isolate allelopathic compounds in Ground elder (*Ageopodium podagraria).* We knew that A. Podagraria probably had allelopathic properties, because of previous studies, but not which the allelopathic chemicals are.
Have you done any similar work prior to the project?
No, not even near the scale of this project. I have only done some minor laboratory work before.
How much literature had you read in order to prepare for the project work?
Since I haven't done any similar work before this, a decent amount of reading was needed to understand what we were doing. I didn't know what GC – MS was prior to this project, for an example.
What did you hope to have as a learning outcome from the project?
How to pursue a real scientific study and how a scientist work.
What did you learn from the project?
Very much, but I'm glad that I got to learn how to pursue a real scientific study, as I was hoping to do.
Is there anything you though was lacking in regard to learning content? Anything you would have liked more focus on?
I found it quite hard to understand why certain methods was used and would have liked a further explanation. The GC MS was hard to understand too.

Did you feel that you got sufficient help and support from the supervisors in areas where you felt uncertain? Did you feel that the supervisors where to involved, not letting you discover solutions to problems by yourself?
I feel that I got the help that I needed but was still let to figure out problems on my own.
What would you say was the most enjoyable part of the project?
The opportunity to pursue a part of it at KTH, it was very exciting and a real experience.
What was the least enjoyable part of the project?
When we had to dig up the ground elder roots…
If this project was to be given to students next year, with the same task as you had, what tips would you give those students in order for them to gain as much as possible from the project?
Read literature and previous similar studies beforehand! It is much more enjoyable if you have enough pre-knowledge to feel that you know what you are doing.
Any other comments?
Thank you, Linus, for great supervision and this amazing opportunity!

Student 3

Describe briefly, in your own words, what the project was about? Aim, hypothesis etc
The aim of the project was to identify and find the allelochemicals in the invasive art Aegodium Podagraria (elder flower). Other previous studies had already shown that the plant may be allelopathic, but the purpose in this case was to identify more specific which compounds that contributes the allelopathic characteristics to A.Podagraria.
Have you done any similar work prior to the project?
No, I have not!
How much literature had you read in order to prepare for the project work?
Since I did not have that much knowledge about allelopathy, I had to read a lot about, for instance, the methods that were used (for example GC-MS) and what allelopathy was all about.
What did you hope to have as a learning outcome from the project?
I wanted to get a broader perspective on what it was like to work at KTH and do a research.
What did you learn from the project?
I have learned more than expected and I have experienced a lot of what it is like to do a more serious research. Moreover, I have learned how to write a scientific report and that sometimes it goes wrong but you have to carry on, be patient and find solutions.
Is there anything you though was lacking in regard to learning content? Anything you would have liked more focus on?
Overall, I think the learning content was good. But it was a lot of own work and sometimes it was hard to understand things. But since we were three in this project, we did much cooperation and we helped each other to understand. Maybe I would have been thankful if we had more specific explainings on why we for instance used MeCN and no other substances?
Did you feel that you got sufficient help and support from the supervisors in areas where you felt uncertain? Did you feel that the supervisors where to involved, not letting you discover solutions to problems by yourself?
I think the supervisors did a great job! We did get the help we needed, but we also did discover solutions to problems by ourselves.
What would you say was the most enjoyable part of the project?
I think most parts of the project was enjoyable. But if I must choose, I would say the part when we came to a conclusion and when we really did understand what the project actually was about and how could be used in future studies.
What was the least enjoyable part of the project?

I would say that the least enjoyable part of the project was to write the report in English since you had to write in a formal and scientific language which we did not had any experiences in. But also we have learned a lot of English which is always good!

If this project was to be given to students next year, with the same task as you had, what tips would you give those students in order for them to gain as much as possible from the project?

To future students, I would say that give it time, be patient and the most important thing; READ READ READ!!!! Do research on your own and try to understand the methods.

Any other comments?

I am very thankful for this opportunity and all the knowledge I have gaine

Table 1S. Summary of the number of analyses performed on each sample. Due to student time limitations, samples were randomly selected to be run in GC-MS in triplicate or as a single run. KRR denotes extracts from *Aegopodium podagraria*.

	0.5 g/mL			1 g/ml		
Sample	**Round 1**	**Round 2**	**Round 3**	**Round 1**	**Round 2**	**Round 3**
KRR 1 Elution I	**3**	**3**	**1**	**1**	**3**	**1**
KRR 2 Elution I	**3**	**3**	**1**	**3**	**1**	**3**
KRR 3 Elution I	**1**	**1**	**3**	**1**	**1**	**1**
KRR 4 Elution I	**1**	**3**	**3**	**3**	**1**	**3**
KRR 5 Elution I	**3**	**1**	**1**	**1**	**3**	**3**
Control Elution I	**1**	**1**	**3**	**3**	**3**	**1**
KRR 1 Elution II	**1**	**1**	**3**	**3**	**1**	**3**
KRR 2 Elution II	**1**	**1**	**3**	**1**	**3**	**1**
KRR 3 Elution II	**3**	**3**	**1**	**3**	**3**	**3**
KRR 4 Elution II	**3**	**1**	**1**	**1**	**3**	**1**
KRR 5 Elution II	**1**	**3**	**3**	**3**	**1**	**1**
Control Elution II	**1**	**3**	**1**	**1**	**1**	**3**

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Response to Editor's and Reviewers' comments and questions

Editor

The editorial team also noted that this work seems better suited as a laboratory experiment - which means that it should be clear that the experiment has been run more than once since this reports the results of working with three students.

Response: We have not changed contribution type to laboratory experiment since the three students worked together as a group and not separately. The experiments performed in earlier years differed between years as explained in the main text and in Supporting Information.

GENERAL:
1. In the submission form, please include the list of all authors as they are listed in the manuscript, names matching exactly.
Response: Authors were added on the submission site, but the high school students do not have official email addresses so dummies were added. Moreover, changes made in the Author profile did not transfer to the submission site.

2. The corresponding author must have an ORCID iD connected with their Paragon Plus account.

Response: The corresponding author has been changed from Linus Svenberg to Åsa Emmer. The ORCID server was not available to connect to despite several attempts at different days. ORCID iD is 0000-0002-3444-9987.

MANUSCRIPT:
1. Under the author list, please provide author affiliations, each with a full address, including, where possible, the department and institute (or company name), city, state/region, postal code, and country.
Response: Full addresses with labels for all authors have been added.

2. Please include all the author names in the references, replacing "et al." (7 and 21).

Response: All authors names have been included.

SUPPORTING INFORMATION:
1. In your Supporting Information for Publication files, please provide a title page with the following: a header, titled "Supporting Information"; followed by the manuscript's title (in title case); author names; author affiliations (including postal codes); and the corresponding author's email address marked with an asterisk.

Response: A title page has been added according to the instructions.

2. Any Supporting Information file included should have an English translation.

Response: Since there are no full translations of the previous high school student reports these have been removed except for the English abstracts, and the latest report which is the base for this study, originally written in English. A summary of the iterative progression through the years have been added in the Supporting Information.

3. Please remove/cover/blur all logos.

Response: All logos are removed.

Reviewer 1

1. This experiment was designed for high school students. Whether the students have a professional background related to GC-MS instruments?
Response: The students were high school students with no prior experience of GCMS. More background about the students has been added in the Background section.

2. How many weeks (or laboratory classes) does the experiment take to complete?

Response: The complete three-year Natural Science program is 2500, and the Diploma project is 100 credits including experiments performed at BGYA and KTH, and writing of the report. About one week was spent at KTH working with sample preparation and GCMS analysis. Text about this has been added in the Background section.

3. In Figure 1, the compound 1 (α-pinene) seems not completely separated from the interference peaks (Figure 1E). Can this affect the characterization of α-pinene by GC-MS?

Response: The reviewer is correct in the assumption that incomplete separation complicates MS identification. The separation could be optimized further but with the limitation in the time this was not possible. Nevertheless, the identification was supported by analysis of reference substances and retention indices (RI), as described in the Experimental Section, and in Results and Discussion.

4. In Figure 1, the peak of compound 2 (caryophyllene) should be marked clearly.

Response: Arrows have been added in Fig. 1 to make it clearer.

5. Why the peaks of α-pinene and caryophyllene in the 1 g/mL elution are very weak (Figure 1 B and F)?

Response: Probably these extractions were less efficient than the others. In that case the low concentration analytes may not be detected.

6. The mass spectra of α-pinene and caryophyllene analyzed by GC-MS should better be given in supporting information.

Response: There were no mass spectra in the supporting information.

7. The "Graphical Abstract" should better be more concise. And, a figure about the experimental process should better be added in the main text.

Response: The Graphical Abstract has been simplified, and a figure describing the experimental process schematically has been added (Figure 1).

8. Some "Student Reports" are not written in English. Can these be translated?

Response: No English translations are available for the first reports. The Swedish parts have been removed and only the English abstracts, and the last report written in English are left in the Supporting information.

9. Pictures of A. podagraria and P. pretense seeds in the experiment should better be given.

Response: Photos of *A. podagraria and P. pretense* have been added in Supporting Information.

10. The files about "student handout" and/or PPT for teaching should better be included in supporting information.

Response: Most of the instructions were given orally by the supervisor present during the experimental work. A generic protocol for UAE and SPE was given to the students, though. This has been added in the Supporting Information. The students were also helped with their literature search as described in the part concerning "Student evaluation and development of the project". These references are included in the reference list of the student report S2.

Reviewer 2

The general concept presented in this paper is that an active learning of inquiry-based project can improve student understanding of analytical chemistry and its application in different areas of life science. The basic idea is sound, and while much has been written in recent years on the value of student engagement in learning, it is still of great value to bring specific activities to the attention of a wide audience. Unfortunately, the presentation in this paper in its current format leaves much to be desired, and would be of limited utility to someone trying to implement a similar strategy in their own course.

Response: A major revision has been performed according to the comments and questions from the editor and reviewers.

Reviewer 3

1.The reported results are from a limited number of analyses: three students in one academic year (I do understand that there are five years of results from prior years lead up to the reported study results)

Response:
Abstracts from the earlier years can be found in Supporting Information. A summary of the iterative methodology development has been added.

2. The authors don't place this in the high school or undergraduate curriculum, which is full of important lab activities, so I think they should indicate if this activity was in place of other normal lab experiences or an additional experience – if it is to fit in the curriculum in place of other labs, what kinds of other lab activities might be roughly equivalent? For example, in an environmental chemistry undergraduate lab, it could replace iron extraction from soil.

Response: More background on the high school program and students has been added in the Background section.

3. I'm not sure the authors clearly concluded whether the 0.5 or 1 g/mL extraction were better, would they suggest one or the other or perhaps a different extraction?

Response: There were no significant differences. A comment on this has been added.

4. There are confusing differences in the width/peak shape of the heptyl acetate internal standard (Fig. 1). Is the entire asymmetric starred peak integrated In Fig. 1C? In Fig. 1F there is a nearly fully resolved peak just before the starred peak; what is the difference in these chromatograms and how does analysis account for it? How does the peak integration deal with the variable peak width? This is critically important to the normalized peak areas and there is no mention of it in the discussion. Also, the peak labeled 2 is small and near other peaks – it should be made clear for example in Fig. 1E that peak 2 is only the first of the three peaks near 15 minutes retention time (if I understand it correctly).

Response: A comment on the peak identification and integration function chosen is added in the Experimental section. This was chosen so that the nearly resolved extra peak should be identified as a separate peak.
Peak 2 has been marked with arrows in Fig. 1.

5. I wonder if there are other reasonable controls to use besides neat hexane (Fig. 4)

Response: Blanks/controls of all solvents used and combinations of these could have been used if time had allowed

6. The Adaptions section needs to give some indication of whether there are reasonable extensions to other allelopathic relationships – in other words, will this only work with Ground Elder and Timothy?

Response: A sentence about the general applicability of the methodology has been added in the Adaptions and Conclusions section.

Editorial comments
1. The authors use several terms in the first two sentences of the Background – I think this makes the beginning of the paper weak, unlikely to grab the reader and make them want to read more – terms like allelopathy, allomones, semiochemicals are unfamiliar; they should start with plant defense processes or communication and then introduce these terms.

Response: Text has been added in the beginning of the Background section to introduce the chemical communication of plants.

2. The article title and abstract need to be adjusted accordingly to the previous item, since the allelopathic effect is unlikely to be widely recognized by the audience they hope to attract

Response: The title has been changed as suggested, as has the abstract.

3. Since European, Scandinavian, and American educations systems can be different in terms of the level of education of a 'high-school' student, there should be some mention of how these systems compare. For example, what is the level of education experience of the three selected students in the study?

Response: Text on the background of the students and their educational program has been added in the background section.

4. The manuscript should be clearer about whether the students or the mentor does certain tasks – I understand that the GC-MS work is done at KTH, understandable since BGYA would not have this kind of instrument. But did the students do the sample preparation at KTH? There is a mention that all analyses were performed by the students – but analysis could mean different levels of hands-on involvement. The methods section indicates the temperature program is 28 minutes, but I don't think it mentions whether the samples are manually injected or via autosampler – are the students sitting there watching the instrument in between injections? If the students are doing sample prep and manual injection, is there a control in place to check for operator consistency?

Response: Some text on the work load of the students has been added in the background section. Some clarifications have also been added in the Experimental section. It was noted in the Experimental Section that a MultiPurpose Sampler (MPS) (Gerstel) was used for injection but it has been emphasized that this is done automatically.

5. The Methods section gives very good detail, but for those considering adopting/adapting this activity, there needs to be some attention to whether any of these details are critical to the success of the method. For example, there is specified a temperature ramp of 12°C/s, is this a usual setting for GC-MS analysis or crucial to getting the results as shown in the figures? Parafilm is mentioned for sealing PP tubes, how important is that? I was not sure if the description indicated that BGYA has a –80 freezer, is that critical or would a regular freezer work?

Response: Comments about this have been added in the Experimental section, and a reference added. Parafilm was used as an extra means to avoid evaporation. No tests have been performed to check how much this influences evaporation.

6. For a manuscript under review – it is annoying to have a figure caption split across two pages (this happens twice)

Response:
The revised manuscript has been checked for this kind of mistakes but there are unforeseen side layout changes when the files are merged into the pdf file on the submission site, which we have no influence on.

7. R&D Part 1, first sentence says third replicate, but Fig 1 caption says replicate 2.

Response: It should be replicate 2, the text has been changed accordingly.

8. I don't know what the term 'area quotes' means.

Response: Area quotes should be area ratio. This has been changed.

9. Page 10 – it should be emphasized that although Fig. 3 omits the 0 results, these are included in the calculated average sprout length, which explains why the mean displayed in the figure is not visually the mean of the displayed histograms in the figure

Response: The text has been changed to make clear that seeds that did not sprout are included in calculations of the average values as 0 cm.

10. The authors don't mention whether the measurement bias to whole centimeters was discussed with the students – a good teachable moment, perhaps. Would the measurements come out differently after students were made aware of this?

Response: This was not discussed with the students, but is a good suggestion for similar projects in the future.

11. Figure 4 should include uncertainty bars

Response: Bars representing min and max lengths of the sprouts have been included.

12. The Associated Content list needs to describe what results are reported in the five reports prior to 2021 so that the reader can decide if it is worth the effort to seek them out.

Response: A summary of changes from the previous reports has been added in the Supporting Information. The reports are however written in Swedish except for the last one, and are therefore omitted except for English abstracts.

13. Are there other documents, such as instructions to students that can be included in the Assoc Content?

Response: A general protocol for the UAE and SPE execution has been added to the Supporting Information.

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30-Jul-2022

Journal: The Journal of Chemical Education
Manuscript ID: ed-2022-00254m.R1
Title: "Investigation of the chemical inhibition effect of Ground Elder (Aegopodium podagraria) on Timothy (Phleum pratense) – Introducing high school students to analytical chemistry and chemical ecology"
Manuscript Type: Article
Author(s): Svenberg, Linus; Malm, Lovisa; Abdollahzadeh, Narin; Gohari, Negar; Almlöv, Maria; Norin, Annika; Emmer, Åsa

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Dear Dr. Hakim,

Thank you for agreeing to review Manuscript ID ed-2022-00254m.R1, entitled Investigation of the chemical inhibition effect of Ground Elder (Aegopodium podagraria) on Timothy (Phleum pratense) – Introducing high school students to analytical chemistry and chemical ecology, for the Journal of Chemical Education. I greatly appreciate your help and look forward to receiving your comments by 13-Aug-2022.

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Author(s): Svenberg, Linus; Malm, Lovisa; Abdollahzadeh, Narin; Gohari, Negar; Almlöv, Maria; Norin, Annika; Emmer, Åsa

Dear Dr. Hakim:

Thank you for submitting your review of this manuscript. Your time and expertise are greatly appreciated and your comments will help us make a decision regarding its publication in the Journal of Chemical Education.

We appreciate the voluntary contribution that each reviewer gives to the journal, and we thank you for your participation in the online review process.

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Thank you.