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REPORT

How do plants protect themselves from insects? A practical laboratory exercise to illustrate the defence mechanisms of the plant through secondary metabolites

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ABSTRACT

Despite the growing awareness of the importance of plant secondary metabolites in insect-plant interactions, undergraduate degree content in agronomy and biology generally does not provide a clear concept to students in relation to secondary metabolite induction of plant defences, implying that students do not obtain a good understanding of the secondary metabolism or its functions. To address this deficiency, we have designed a practical exercise where students determine the phytochemical induction of secondary metabolites in aromatic plants subjected to herbivory. This approach involves an experimental laboratory class in which students evaluate the phenolic compounds and main essential oil compound induction in peppermint damaged by an armyworm. By the end of this exercise, based on the results and findings, students will: have a better comprehension of plant defence responses to herbivores; be able to illustrate the consequences of insect herbivory in relation to plant secondary metabolites induction; acquire lab skills related to the use of a spectrophotometer; be able to understand and analyse a GC chromatogram report. Authentic research experiences in the classroom are considered valuable elements for promoting science at undergraduate level, as well as providing motivation for the student and linking research with teaching.

KEYWORDS

Herbivory; insect-plant interaction; secondary metabolites; experimental laboratory class; applied learning

Introduction

Plant-insect interactions are classically viewed as mutualistic, antagonistic, or commensalistic. Mutualism is characterised by reciprocal benefits provided by each partner, with both profiting and neither being harmed. Examples of mutualism include pollination (e.g. flowering plant/insect pollinator systems), plant guarding, or seed dispersal. In contrast, in antagonistic relationships, one partner benefits and the other is harmed, while in commensalism, one counterpart benefits but the other neither benefits nor is harmed (Calatayud, Sauvion, and Thiery 2018).

Plants have evolved an enormous array of mechanical and chemical defences against herbivores. Plant-insect interaction is a dynamic system, which is subject to continual variation and change. In order to reduce insect attacks, plants have developed different defence mechanisms. Resistance factors for direct plant defence against herbivorous insects comprise plant traits that negatively affect insect preference (host plant selection, oviposition, feeding behaviour) or performance (growth rate, development, reproductive success), resulting in increased plant fitness in a hostile

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environment. These traits include morphological features for physical defence, such as thorns, spines, and trichomes, and also incorporate chemical defence, involving secondary metabolites (SM), digestibility reducing proteins, and antinutritive enzymes. All of these traits may be expressed constitutively, as in preformed resistance factors, or they may be inducible and deployed only after attack by insect herbivores. Furthermore, the induction of defensive traits is not restricted to the site of attack, but extends to non-infested healthy parts of the plants (Erb and Kliebenstein 2020).

These strategies are able to deter most herbivores, although there is a reduced number of insects that are able to adapt to specific plant species. SM perform useful functions for the plant, by acting either in an inducible or constitutive manner. Secondary plant compounds are involved in plant defence against insect herbivores by acting as insect repellents, feeding inhibitors and/or toxins, as well as by attracting the natural enemies of herbivores (War et al. 2012). SM are characterised on the basis of their chemical structure, composition, and solubility in numerous solvents, or by the pathway by which they are synthesised (Hussein and El-Anssary 2018; Erb and Kliebenstein 2020).

The series of lab experiments described below are aimed at teaching students from the insect-plant interaction course for undergraduate students, which forms a part of various degree studies including agronomy, biology and biotechnology. To teach students with different backgrounds, we consider it important to create connections between learners' 'past, present and future knowledge and authentic experiences'. Connecting learning with personally, culturally, and socially meaningful and relevant problems, and with scientific development, provides authentic contexts where knowledge can be used in real-life activities, incidents or simulations. Our experience has shown that students taking different degrees have a lack of experimental practice related to phytochemistry. In particular, in the course on insect-plant interaction, the phytochemical induction after insect attack topic is approached in class only by lectures and paper-based activities. Consequently, the students, despite having clear concepts with respect to physical plant defences, demonstrate their uncertainty when asked to explain chemical induction in plant defences. Based on these considerations, we designed a practical exercise to give students a laboratory class where students can determine the phytochemical induction of SM in aromatic plants subjected to herbivory.

Regardless of the fact that the students have previous knowledge of organic chemistry, biochemistry and plant physiology, they do not have a clear understanding of second metabolism. Although the definitions of SM are inherently diffuse (Pichersky and Lewinsohn 2011; Erb and Kliebenstein 2020), the differences between primary metabolites, SM, and plant hormones have found their way into textbooks and shape our thinking in plant biology to this day. An illustrative example is the field of plant–herbivore interactions, where major efforts have gone into disentangling how plants protect their primary metabolites (which serve as nutrients for herbivores) using SM (as defences for plants), and also how adapted herbivores manage to extract primary metabolites while avoiding any negative effects of SM (Howe and Jander 2008; Zhou et al. 2015; Erb and Reymond 2019).

Taking the above into consideration, we have designed an original laboratory experiment aimed at improving student understanding and identification of plant defence responses to herbivory, essentially by reviewing and integrating the concepts taught in previous courses in a somewhat superficial way. This experiment contemplates the study of total phenolic compounds and main essential oil (EO) compound induction in *Mentha piperita* (peppermint) damaged by an armyworm (a third instar larvae of *Rachiplusia nu). R. nu* is a polyphagous noctuid pest endemic to southern South America. The larval stage of *R. nu* can cause substantial damage to crops, especially soybean, sunflower, maize, alfalfa, and tobacco, as well as certain horticultural and aromatic species such as peppermint (Rimoldi et al. 2012). This interaction is suggested because peppermint is an aromatic and medicinal plant cultivated worldwide mainly for its EOs, which are then used in fragrance, spices and the pharmaceutical industries (Jullien 2007; Lubbe and Verpoorte 2011). EOs are complex mixtures, constituted by terpenoid hydrocarbons, oxygenised terpenes and sesquiterpenes. They originate from plant secondary metabolism and are responsible for their characteristic aroma. Peppermint plant extracts also have flavonoids, polyphenols and carotenes, resulting in a high antioxidant activity (Farnad, Heidari,

and Aslanipour 2014). The proportion of phenolic components present in the leaf represent about 20% of the dry weight (Figueroa-Pérez et al. 2014; Riachi and De Maria 2015), Therefore, many medicinal properties of peppermint can be attributed to EOs and antioxidants (Krishnaiah, Sarbatly, and Nithyanandam 2011; Rahimi, Alireza, and Mojtaba 2018).

By the end of this exercise, as a result of obtaining a better understanding of plant defence responses to herbivores, the students will be able to: (a) Illustrate the consequences of insect herbivory in relation to plant secondary metabolite induction; (b) Acquire lab skills related to the use of the spectrophotometer; (c) Understand and analyse a GC chromatogram report; (d) Accumulate skills in the interpretation, discussion and recording of experimental results. From the learning point of view, the approach taken in linking teaching with research is constructed to immerse learners in authentic research experiences, since research in science teaching and learning should involve identifying and asking appropriate questions, designing and conducting investigations, collecting evidence, drawing conclusions, and communicating and defending findings. For this purpose, this practical session was designed with no time constraints (4 days of lab). In this way, the learners have more time for reflection and interaction, both essential factors for 'meaningful learning' to occur (Gunstone and Champagne 1990). However, meaningful learning requires a student to possess some prior knowledge of a topic, in order for the material to be meaningful, and requires that the learner chooses to learn in a profitable way (Bretz et al. 2013). Through performing this laboratory exercise, the students can engage with this form of learning, where the format is modified so that they can construct their understanding based on the results and findings obtained, and thereby be provided with the opportunity to critically evaluate the data and support any conclusions using the evidence obtained (Abidin et al. 2013). This form of meaningful learning occurs across three domains: doing (psychomotor), thinking (metacognitive), and feelings, emotions and attitudes (affective) (Emenike, Daneilson, and Bretz 2011; George-Williams et al. 2019). Finally, students communicate their findings in a written report, and a lab report rubric has been generated to assess the laboratory report in the form of a scientific publication, based on the idea that the process of writing leads to a deeper understanding of developing concepts (Graham and Hebert 2011).

Experimental procedures and implementation methodology

Research practical process

Two techniques were proposed to determine the effect of herbivory on SM induction in peppermint plants, namely total phenolic compound content (TPC) and the concentration of the main compounds of peppermint essential oil (EO) (Figure 1).

The experimental procedure described below was carried out in three lab sessions of about 3–4 hours (Table 1-Figure 2), with simultaneous participation involving no more than twenty students. A fourth session, of about 2 hours, was then required for data recording and the analysis of the results (Table 1). Finally, their findings were communicated in a written report. This laboratory exercise must be planned by the teacher in advance, due to it being necessary to work with *R. nu* 3rd instar larvae starved for 24 h.

Proposed Activities (Table 1)

On day 1:

- The theoretical background was presented and the experiments were organised by the instructor. Aims and the hypothesis of the experiment were defined.
- Working groups were established and the procedures were described.
- The plants were exposed to the *R. nu* larvae by the student

On day 2 (48 h after the first class):

- Vegetal material was collected.

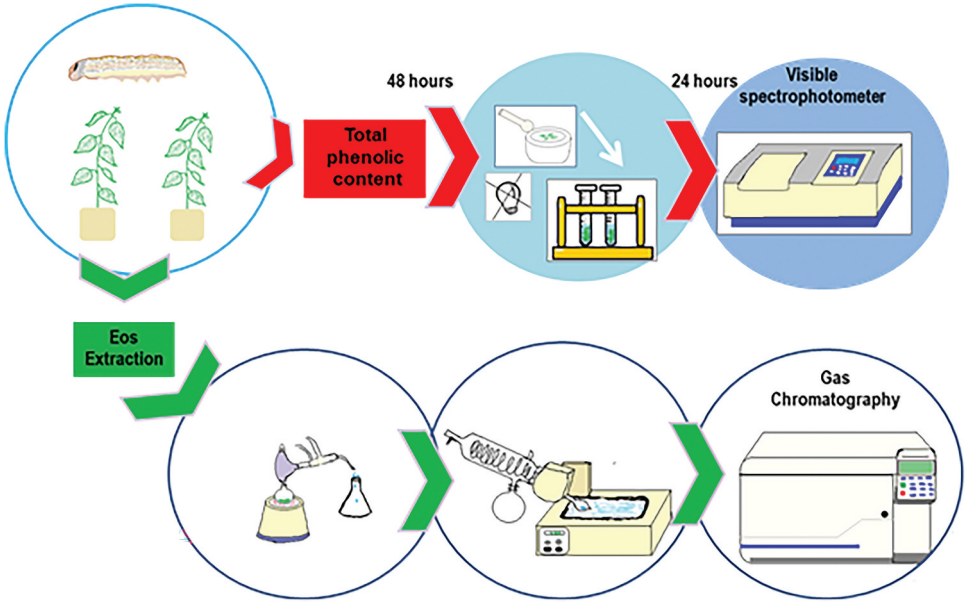

Figure 1. Scheme of the procedure for the determination of total phenolic compound content and essential oil extraction.

Table 1. Laboratory exercise timetable for the different techniques used for the determination of the secondary metabolites of plants damaged by insects.

Day	Time (h)	Activities
1	3	Theoretical explanation. Definition of aims and hypothesis of the experiment. Creation of the two working groups. Description of procedures.Exposure of plants to insects.
2 (48 hs since day 1)	12	TPC procedure:Prepare the tissue for TPC determination (24 h incubation).EO extraction: Hydrodistillation.Concentration and acquisition of EOs.
3 (24 hs since day 2)	22	TPC procedure:Obtain the TPC calibration curve.Measure TPC samples by the spectrophotometric technique.EO extraction: Analysis and interpretation of GC reports. Perform statistical analysis and plotting of graphs.
4	2	Analyse and discuss the results and present a comprehensive written laboratory report.

- The total phenolic compound procedure was explained by the instructor: The tissue was homogenised with distilled water, transferred to a test tube and left for 24 hrs in the dark.
- EOs were extracted by hydrodistilation (second part of the class).

On day 3:

- The standard calibration curve for gallic acid was constructed
- An explanation of how to understand and read a GC report was presented by the instructor.
- Chromatogram reports from the EOs extracted the previous class and provided by the instructor were analysed by the students.

On day 4:

- The results obtained during the previous classes were analysed by the students. This implied combining the results of the two groups. The experimental results were compared with those of the control treatments by performing the required statistical analysis, and the results observed were plotted.

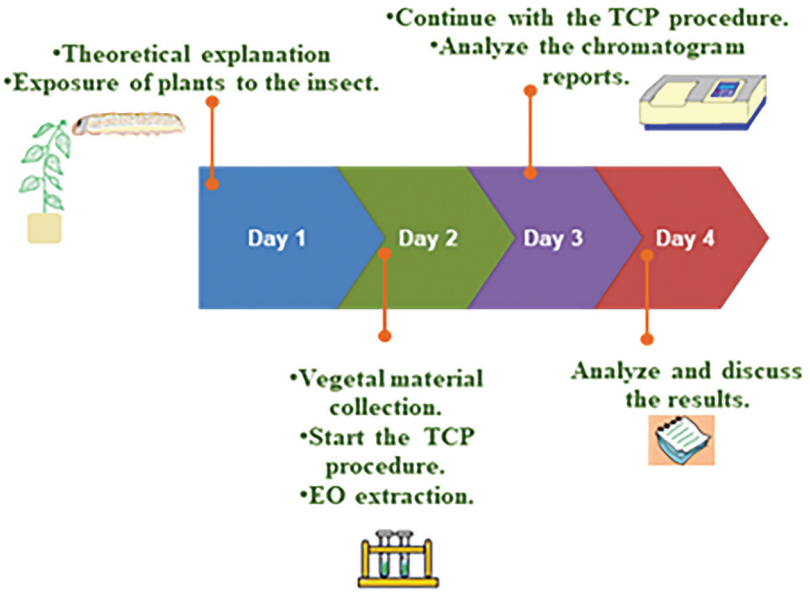

Figure 2. Laboratory exercise workflow.

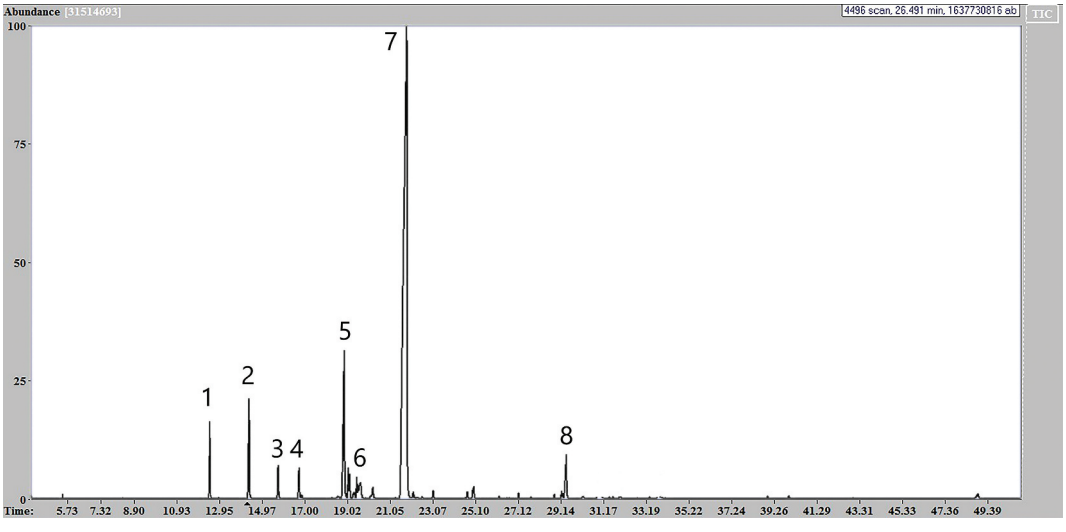

Figure 3. GC-MS chromatogram of *Mentha piperita* L. essential oil.

Insect, plant material, and treatments

To complete the two proposed experiments, the students were separated into two groups, with each group working with control plants (CP) (without insect damage) and insect-damaged plants (IP). As there should be at least 2 plants per group/ per treatment, this scheme required a minimum of 8 plants.

Insect

Rachiplusia nu (Lepidoptera: Noctuidae) is a polyphagous noctuid pest. Approximately, twelve third instar *Rachiplusia nu* larvae starved for 24 h were required.

Plant material

The peppermint plant (*Mentha piperita*) was chosen because it is easy to propagate, as well as being a very fast growing plant. *M. piperita* plants contain ~3% volatile oils consisting of >50 different compounds. EOs, accounting for ~60% of total oil volume, were identified as (+) pulegone, (-) menthone, (-) menthol, and (+) menthofuran (Cappellari et al. 2015). In addition, there was a large number of phenolic compounds (Figueroa-Pérez et al. 2014; Riachi and De Maria 2015).

Treatment

Four peppermint plants were used for the insect treatment (IP). Each plant was exposed to 3rd instar *R. nu* larvae starved for 24 h. The larvae were carefully moved with a fine brush to place three larvae on each plant, without leaving more than one larvae on the same leaf. After 2 h, these larvae were removed. The presence of four larvae for 2 h was found to have caused damage to about 30% of the leaf. Related to this, several studies have revealed changes in SM after 48 hours of herbivory (Zebelo et al. 2016; De Bobadilla et al. 2017; Cappellari et al. 2020). Plants exposed to larvae were then placed in a separate chamber to avoid the possibility of volatile compounds influencing the plants of the other treatments.

After 48 hrs of insect damage, the leaves were cut from the plants at the base of the petiole with a scalpel from IP and CP (control plant). All the leaves from each plant were then weighed and placed separately in aluminium foil and labelled. Students were required to use at least two plants from each treatment, with 200 mg for each plant being used for TPC and the rest of the plant used for EO extraction.

Determination of total phenolic contents (TPC)

The total water-soluble phenolic content was determined using the Folin-Ciocalteu colorimetric reagent (Singleton and Rossi 1965). This method is based on the capacity of phenolic compounds to react with oxidising agents. The Folin-Ciocalteu reagent contains molybdate and sodium tungstate, which react with any type of phenolic compound to form a phosphomolybde-posphotungsic complex (Peterson 1979).

To determine the TPC, a calibration needed to be obtained. To do this, different volumes were taken from a standard solution of gallic acid (1 mg/ml), which were completed with a corresponding amount of DW. Then, the optical density (OD) was calculated at a 760 nm wavelength, with the resulting data plotted and analysed using regression statistics utilising supplied software packages such as Excel (Chiappero et al. 2020).

TPC procedure: Plant tissue (200 mg) was homogenised in a mortar with 5 ml DW, and then transferred to a test tube and left for 24 hours in the dark (and covered with aluminium foil). The next day, 0.5 ml of the supernatant plant extract was carefully transferred to a test tube, and 8 ml DW and 0.5 ml of Folin-Ciocalteu reagent were added. After 5 min, 1 ml of Na_2CO_3 (20% p/v) solution was added, and after a further 1 h, the TPC level was determined by colorimetry at a wavelength of 760 nm (complete Table 2). Finally, the calibration function was used to estimate the TPC values expressed in terms of mg gallic acid (a common reference compound) equivalent per g plant fresh weight (FW) (Figure 1) (Cappellari et al. 2013).

Essential oil extraction

Leaf samples from the rest of whole plant used for TPC determination were individually weighed and placed in a 100 ml round bottom boiling flask with 50 ml distillated water (DW). The material was then subjected to hydrodistillation using a Clevenger-like apparatus for 40 min. In order to separate the EOs from the aqueous layer, the hydrodistilation volume was introduced into

Table 2. Sample preparation solutions used for Total Phenolic Compound (TPC) determination.

		DW (µl)	Crude extract (µl)	Folin-Ciocalteu reagent (µl)	Na_2CO_3 20% (µl)	Abs 760 nm	[TPC] in the sample
	Blank	8500	-	500	1000		
CP	CONTROL 1	8000	500	500	1000		
	CONTROL 2	8000	500	500	1000		
IP	I 1	8000	500	500	1000		
	I 2	8000	500	500	1000		

a separation funnel and 20 ml dichloromethane were added while working under an extraction hood and using nitrile gloves. The dichloromethane fraction was then collected and a small amount of the drying agent was added from the tip of a spatula (Na_2SO_4) to extract any water that could be inside this fraction. Next, β-pinene (1 µL in 50 µL ethanol) was added as an internal standard (β-pinene was reported not to be present in peppermint plants; Cappellari et al. 2015) to the dichloromethane fraction and transferred to a boiling flask. The solvent was removed with a rotary evaporator at 40°C, and the EOs were collected and stored at 4 °C until being analysed (Figure 1) (Cappellari et al. 2015). Considering that only a small amount of EO can be extracted from one peppermint plant, it was suggested to the students, at this moment, to leave a volume of approximately 300 ul when the solvent was dried.

Gas chromatography

The development of chromatographic techniques has allowed the chemical composition of essential oil to be determined. Gas Chromatography (GC) is without doubt the best method currently available, due to its simplicity, rapidity and efficiency for both the identification and quantification of essential oil components and composition variations.

Separation and identification of the main eluted components were achieved by chromatography mass spectrometry (GC-MS). Although GC/MS is the most powerful method to carry out both the qualitative and quantitative analysis of natural compounds of a complex composition, the quantitative method carried out here used GC/FID, as it is more generally accessible in laboratories.

The EO components were identified by students using a gas chromatograph with a selective mass detector (GC/MS), a CBP-1 capillary column (30 m x 0.25 mm, film thickness 0.25 µm), a mass-selective detector and a split/splitless injector. The analytical conditions used were: injector and detector temperatures of 250 and 270°C, respectively; oven temperature programmed from 60°C (3 min) to 240°C at 4°/min; carrier gas = helium at a constant flow of 0.9 ml/min; source 70 ev. The individual peaks were identified by comparison of their retention indices (RI) with those of authentic samples, as well as by comparing their mass spectra with a library by spectrum matching (Library NIST Mass Spectral Search Program) (Cappellari et al. 2015).

Analysis of EO components

The major components identified in the essential oil of *M. piperita* of plants contained ~3% volatile oils, which consisted of >50 different compounds. The principal EO components identified, which comprised ~60% of total oil volume, were limonene, linalool, (−) menthone, (−) menthol, and (+) pulegone (Cappellari et al. 2015). However, the composition of these oils differed in their main components compared to data reported from other sources, probably due to differences in growth conditions (Figueiredo et al. 2008). When extracting the EO of a small amount of biomass, only the main components were registered in the GC analysis. Thus, only (−) menthone, (−) menthol, and (+) pulegone, the three major components, were considered in the present assay.

Identification and quantification of EO main components

In order to quantify a component by GC, an internal standard was used. This allowed us to calculate the concentration or percentage of mass of one or more of many constituents that appeared as being separated in the chromatogram, even in the presence of unsolved peaks. The known volume of that which had been diluted was injected, and the reference area (Aref) and the corresponding peak in the chromatogram were measured. Then, the area corresponding to the sample (Asample) was measured. Since there was proportionality between the areas, which depends on the injected masses and the concentrations, the sample concentration was determined using the equation (Rouessac and Rouessac 2003)

$$\text{Sample concentration} = \frac{\text{Asample}}{\text{Aref}} \text{ x reference concentration}$$

Chemical analyses were performed using a Perkin-Elmer Q-700 gas chromatograph (GC) equipped with a CBP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 μm) and a mass selective detector. The analytical conditions used were: injector temperature 250°C; detector temperature 270°C; oven temperature programmed from 60°C (3 min) to 240°C at 4°/min; carrier gas = helium at a constant flow rate of 0.9 mL/ min; source 70 eV. Oil components (limonene, linalool, (−) menthone, (−) menthol, and (+) pulegone) were identified by comparison of the diagnostic ions (NIST 2014 library) and the GC retention times with those of respective authentic standard compounds purchased from Sigma-Aldrich (St. Louis, MO, USA) (Cappellari et al. 2015) (Figure 3-Table 3).

Timing and student participation

Before the first laboratory day, students were required to read a worksheet that introduced the topics and activities to be covered. The instructor was responsible for preparing the peppermint seedlings and larvae in advance. On the first and second days, the students developed the experiment and prepared the samples for the extraction of the EOs and TPC (Table 2). They worked in two separate groups throughout the laboratory exercise, and on the third laboratory day they finished the TPC analysis and used the spectrophotometer. For the GC analysis of the EOs, it is suggested that the instructor performs the GC-FID analysis, identifies the main compounds, and gives the GC reports to the students with the identification of the retention time of each of the main compounds to be analysed. This was proposed as the injection of all the samples would require too much time. In addition, a brief explanation was given by the instructor on how to interpret the GC report, after which, the students were able to analyse the GC report.

Finally, students were required to write a report structured like a scientific publication, for which guidelines to follow were given. They were expected to be able to analyse their results, reach conclusions and decide whether more experiments were needed, as well as to mention any new

Table 3. Composition of the essential oils of *Mentha piperita* leaves. Individual areas, their addition and the relative percentage of each component, corresponding to the chromatogram in Figure 2.

Number of peak	Retention Time (min)	Area	Relative Area%	Compound
1	12.306	2,388,964.500	3.78	**β-Pinene (standard)**
2	14.337	3,991,940.250	5.82	limonene
3	15.738	2,899,162.500	4.36	Sabinenehydrate
4	16.703	795,255.375	1.56	.β-Linalool
5	18.804	8,758,175.000	12.77	**Menthone**
6	19.449	1,713,141.750	3.78	**Menthol**
7	21.635	48,495,008.000	65.19	**Pulegone**
8	29.153	1,459,292.375	1.94	Viridiflorol

questions that may have arisen during the course of the investigation. The written report was evaluated using a rubric (Table 4), and the qualifications used for the degree of success achieved were descriptive (proficient, adequate, substandard, unacceptable), thereby avoiding the use of letters representing grades or numbers representing marks. Rubrics helped the students to recognise learning goals and guided them to reach those goals. Being particularly useful in research-based learning, rubrics have been used to assess content mastery, skill development, or even attitude towards a topic (Mullinix 2014). Furthermore, rubrics help teachers to teach, aid in coordinating instruction and assessment, and also help students to learn. To write or select a rubric, teachers need to focus on the criteria by which learning will be assessed. This focuses on what the instructor intends students to learn, rather than on what the instructor intends to teach, thereby helping to improve instruction. Rubrics provide clarity of both the content and outcomes (Fitzgerald and Byers 2002; Reynders et al. 2020).

Safety considerations

Students were required to wear a laboratory coat and gloves for the experimental tasks. There were no biological risks associated with these assays. When working with dichloromethane (see Essential Oil Extraction section), students were instructed to work in a fume hood. Our institution has compiled its own Laboratory Advisory Guidelines that provide procedural information for laboratory workers about how to dispose of laboratory waste. These guidelines were read in conjunction with Hazardous Waste Disposal Guidelines, in order to minimise risks.

Statistical Analysis

Data were subjected to a t-test analysis. The differences between the means of treatment and control were considered to be significant for p values < 0.05.

Results and discussion

In the second class, 48 h after larval damage, students were instructed to remove the leaves from plants and used this fresh material for TPC and EO yield valuation. In the third class, the students used the calibration function from the gallic acid curve to estimate the TPC and to express the TPC content in terms of mg gallic acid per g plant fresh weight using:

TPC mg gallic acid/g FW = [TPC]/0.2 g (as 200 mg is the weight of the peppermint leaf used).

The amount of the main EO compounds, menthol, menthone and pulegone, were obtained based on the GC report.

For the statistical analysis, students performed a t test analysis with all data obtained from the different parameters evaluated, and then the data from both groups were analysed together. Next, a graph showing the effect of insect herbivory using the data obtained from both groups was plotted. Related to this, plants exposed to larvae damage were expected to have a significantly increased TPC, and also increased amounts of menthone, menthol and pulegone present in the peppermint EOs (Cappellari et al. 2020). The results were discussed considering the theory explained above, and any atypical results were debated with respect to possible effects of errors made during the procedure.

It was expected that students analysed their results, reached conclusions and decided whether more experiments were needed, as well as identifying any new questions that may have arisen during the course of the investigation.

It was important to make students aware through discussion that the effects of herbivory on plants are complex. The analysis of the activation of the studied mechanism was discussed with respect to the advantages given to the plant. The negative effect of insect herbivory was also

Table 4. Lab report rubric.

Item	Proficient	Adequate	Substandard	Unacceptable (Fail)
Introduction:Objectives	Raises, exposes and explains synthetically and clearly all proposed objectives, recognising the potential and limitations of the work done.	Raises, exposes and explains adequately, but not always clearly, the proposed objectives.Recognises the potential and limitations of the work done.	Raises, exposes and explains only partially the objectives proposed, and without recognising the potential and limitations of the work done.	Raises and exposes only the objectives.
Introduction:Theory	Recognises and exposes in a clear and synthetic way the theoretical concepts on which the objectives based, and those on which the experiment is carried out.	Recognises and exposes adequately, but not always clearly, the theoretical concepts on which the objectives are based and those on which the experiment is carried out.	Recognises and exposes only some of the concepts, and reveals a lack of understanding of them.	Does not understand or know how to apply the concepts or theories in the work carried out.
Material and Methods: ExperimentalProcedure	The procedures to be followed in the lab practice are described and listed in a clear and precise way as being optimal for achieving the objectives of the topic analysed.	The procedures to be followed in the lab practice are reasonably well described and listed, and these are recognised as being consistent for achieving the objectives of the topic analysed.	The procedures to be followed in the lab practice are described and listed.	Does not identify, describe or list the procedures to be followed in the laboratory practice.
Material and Methods: Materials and Instrument Management	Describes all the elements to be used. Optimises and uses creatively the resources. Identifies that the material used is necessary to clearly address the problem analysed.	Describes the elements to be used, but these are not used optimally or creatively. Some missing materials impede an approach with clarity of the problem analysed.	There are deficiencies in the material presented and in its mode of use.	Describes but misuses or does not use optimally the items. Missing materials prevent an acceptable solution to the problem to be analysed.
Material and Methods: Experimental data handling	Presents the data obtained in an orderly way with tables, figures, diagrams, etc. All the data obtained have relevant indications and observations.	Presents the data obtained in orderly way with tables, figures, diagrams, etc. However, not all the data obtained has relevant indications.	Presents the data obtained in an unclear way. Not all the data is relevant.	Does not present data, or these are inaccurate and presented in an unclear way.
Material and Methods: Analysis of data	Uses and presents in a synthetic, clear, and critical way the equations, graphs, and diagrams or figures which relate to the problem analysed. Explains the possible data errors. An optimal processing of the data and information is achieved.	Uses and presents reasonably well the equations, graphs, and diagrams or figures which relate to the problem analysed. Explains the possible data errors. A consistent processing of the data and information is achieved.	Uses and presents in an incomplete, inaccurate, or unclear way the graphical equations, and diagrams or figures which relate to the problem analysed. Does not explain possible data errors. Only achieves a partial processing of data and information.	Does not present equations, graphs, or figures that are related to the problem analysed. Does not achieve processing of data or information.
Results:Connection observation – theory	Associates clearly and in a precise and relevant way the problem analysed with theories.	Associates reasonably well the problem analysed with theories.	The connection between the problem analysed and theory is imprecisely stated.	Fails to connect the observations obtained with the theory.
Conclusions	Draws consistent conclusions argued with the generation and synthesis of the new knowledge for the problem analysed with respect to the objectives and the case study carried out.	Draw partial conclusions argued with the generation and synthesis of the new knowledge. However, does not show coherence when connecting the problem analysed with the case study and the objectives.	Draws conclusions that do not explain the phenomenon analysed.	Unable to draw conclusions.

(*Continued*)

Table 4. (Continued).

Item	Proficient	Adequate	Substandard	Unacceptable (Fail)
Critical thinking	Analyzes, evaluates, or combines relevant information to form an argument or reach a conclusion supported with evidence. Suggests improvements.	Partially analyzes, evaluates, or combines relevant information to form an argument or reach a conclusion, but with some errors.	Only minimally evaluates, or combines relevant information to form an argument or reach a conclusion, and with some errors.	Inaccurately evaluates or combines relevant information to form an argument or reach a conclusion.
Bibliography	Identifies the sources of information used.	Uses the bibliography, but does not clearly identify the sources of information used.	Uses the bibliography, but does not identify the sources of information used.	Does not use the bibliography.
Team work	The group is seen to be bonding, and has a consistent idea of what was accomplished.	The group appears to be functioning well, but with not all the members having an equal understanding of what was accomplished.	The group does not appear to function well, with not all members having an equal understanding of what was accomplished.	There is very little evidence of teamwork.

considered here, as it is a multidimensional stress and generally leads to changes in the physiological, morphological, ecological, biochemical and molecular traits of the plant. In addition, it can negatively affect the quantity and quality of plant growth and yield.

It was highlighted that each plant interacts with insects in a different manner, and that insects from the same species may produce different effects on plants depending on the particular plant species damaged (Erb and Reymond 2019). Also, the evolution of plant defences was discussed in the context that insects were among the earliest terrestrial herbivores and acted as major selection agents on plants. Plants evolved chemical defences against this herbivory, and the insects, in turn, evolved mechanisms to deal with plant toxins (Erb and Reymond 2019).

Finally, students were required to write a report structured like a scientific publication, which had to be handed in within seven days of finishing the laboratory exercise. The opportunity to enhance their scientific writing skills was provided by this final report, which further contributed to the learning process. The inclusion of rubrics in the assessment increased the authenticity and motivation. From the teacher's perspective, this produces greater transparency, which promotes reflective practice and helps provide feedback (Jonsson and Svingby 2007). In addition, it has also been considered useful to make the criteria explicit and to guide the qualifications of the teachers (Allen and Tanner 2006).

Student outcomes

This lab exercise has been used over the last two years on the Insect-plant course as part of a series of laboratory practices in which different insect and plant situations were evaluated, and with the experiments performed being reproducible. The evaluation of student performance was carried out primarily thorough a journal-style written lab report using the lab report rubric (Table 3). The experimental procedure described above could also be adapted for postgraduate courses. It is appropriate to point out here that agronomy and biology students had taken the organic chemistry and biochemistry courses in their first and second years. Both these courses provide students with a comprehensive background for subsequent courses such as insect plant interaction, but students of these different degrees have a lack of experimental practices focusing on phytochemistry.

In terms of the curriculum-teaching plan, students are provided benefits by this laboratory exercise, in addition to learning the content of the course, which include the possibility of practising again methodologies previously learned on previous courses, as in the case of the spectrophotometry technique. Related to this, in the biochemistry course, one laboratory exercise carried out is the determination of the amounts of protein in a biological sample by spectrophotometry. Although students have a worksheet where the background of the technique is explained, they only have to set up the reagents in the sample to obtain the colouring, as well as measuring the optical density. Through a survey carried out on the students, it was discovered that they have difficulties in relating the theory to the practical exercise where they learn the spectrophotometry technique, with students indicating that they had performed the measurements without completely understanding the scope of the methodology for certain biological situations. Thus, the laboratory exercise improved their knowledge about this topic.

In relation to the analytical technique (the gas chromatography analysis) which allows the separation and identification of organic compounds, in the organic chemistry course this was only mentioned in the lectures, with students not having any practical example of this technique. In the present lab experiment, although students do not personally use the chromatography equipment, they obtained a better understanding of the principle of gas chromatography and how to prepare a sample, and also learned how to interpret the GC report.

In this lab practical, the use of statistics was particularly emphasised since the students stated that they completed the statistics course but never applied it to any practical activity, and also that when they are developing their postgraduate studies they do not know how they should use the knowledge previously acquired.

The students' perception regarding the lab report rubric was that the evaluation method was more transparent and fairer, as it clarifies the objectives and reduces uncertainty by making critical aspects of the task clearer. This allowed students to regulate their processes and obtain immediate feedback, which was associated with a decrease in anxiety and increased satisfaction with the evaluation.

At the end of the insect plant interaction course, students were asked to fill out a survey through the university platform UNRC-EVELIA in order to assess the impact of this lab activity on the student's comprehension. In particular, students highlighted that by having a visual experience they achieved a better understanding of the theoretical concepts. The feedback obtained from students in relation to this practical activity was focused on the extraction and concentration equipment, and on the processing technology for the study of EOs. Participants expressed their surprise at how easily essential oils could be obtained, which they had never considered before. And they also now realised that EOs are used in a wide variety of consumer goods, such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages and insecticides. They also mentioned that they were now aware of the fact that the world production and consumption of essential oils and perfumes are increasing very fast.

Finally, the students reported that they had made significant gains in skills that were not directly emphasised by the instructor on the course, which included improving their communication skills, both among team members and through the writing of a common, comprehensive laboratory report. Thus, the results indicated that the realisation of practical laboratory activities carried out in groups was a good educational technique for improving inter-student communication practices.

Conclusions

The proposed experimental method to investigate the effects of herbivory on SM of an aromatic/medical plant was designed as a practical lab session and followed a typical scientific experimental procedure, in which students collect and analyse their results and then determine whether their findings support or not the hypotheses discussed during the theoretical part of the course. In the field of science education, this provides the teacher with the opportunity of introducing new instructional methodologies that favour research and knowledge creation (Vanaki and Memarian 2009). Authentic research experiences in the classroom are considered valuable elements for promoting science for undergraduates, through motivation of the student and linking research with teaching (Wilson, Howitt, and Higgins 2016). The series of lab experiments described was an approach to modifying existing practical activities to promote critical thinking in students, thereby supporting enhanced learning. Notably, in other laboratory classes, where the learner translates theory to practice, students do not consider the significance of their results. In the present lab experiment, much more than just processing information, evaluating, interpreting, and manipulating or transforming of information are required. In this exercise, students were asked to analyse the data they collected, combine data from different sources, and generate arguments or conclusions about their data, considering this as critical thinking. However, in contrast, when students simply follow the so-called 'cookbook' laboratory instructions that require them to confirm predetermined conclusions, they do not engage in critical thinking (Reynders et al. 2020).

This lab experiment aids students in integrating theory and practice, ensuring that they have a better understanding of the relation between both these elements, and also motivates students inclined towards scientific research to consider this possibility for their future career. In summary, this experiment allowed students to directly assay SM induction in plants after herbivory through the use of different methodologies such as spectrophotometry, and in particular, the extraction and analysis of EOs. It should be highlighted that the extraction and analysis of EOs is a topic that

students have not addressed during the degree course and that students found it very exciting and useful. Through performing the activities proposed here, methodologies addressed in previous courses are complemented and reinforced.

In recent years, there has been an increasing interest in the use of aromatic medicinal plants and EOs. However, the rise in the consumption of the available natural resources has become a new challenge and a common worldwide approach is needed. Moreover, the emerging trend of a return to nature has caused a preference shift from synthetic to herbal, natural, and traditional applications (Gunjan et al. 2015). It is therefore important to create an interest in the students concerning these novel topics, which are currently the focus of increasing research effort (Bell 2011; Gasper and Gardner 2013). This will increase student understanding of the nature of state of the art scientific research, and can consequently lead to a greater enthusiasm in the students (Gasper and Gardner 2013).

Rubrics have been developed to characterise the level of inquiry in laboratory exercises (Buck, Bretz, and Towns 2008; Fayer et al. 2011), and it is well established that student perceptions of practical work improve when active learning strategies in are adopted (Emenike, Daneilson, and Bretz 2011; Kirkup et al. 2010). Indeed, it is widely accepted that engaging students in authentic processes of scientific enquiry, including both in laboratory and post-laboratory writing (Moskovitz and Kellogg 2011), will motivate and engage students with varying interests and abilities and from diverse backgrounds (Handelsman et al. 2004). It has been reported that the way in which the evaluation is carried out has implications for the learning process of the students. Concerning this, it has been reported that teachers who perceive teaching as solely the transmission of knowledge consider assessment to be a separate element of teaching, rather than as an integrated strategy that promotes deep learning. On the other hand, those who perceive teaching as knowledge construction adopt different evaluation practices, in addition to the traditional ones. This is relevant, since the assessment methods used by university teachers have an important role to play in the quality of learning (Ribeiro and Assunção 2016).

Scientific disciplines should be taught using active methods focused on application (Waldrop 2015), partly through experimental methods to contribute to the acquisition of procedural competence, and also to improve problem solving, academic performance and social interaction (Ifeanyichukwu 2016). Therefore, taking into account the authentic evaluation model, we consider that in order to contribute to the training of scientists, some of the tasks that they will face must be incorporated into the classroom of the future professional. These include the design of experiments, data analysis, execution of experiments, communication of results at scientific congresses, preparation of laboratory reports, and the writing of scientific publications and research projects. Although some of these activities are generally included in undergraduate subject programmes, they tend to have a low weighting in the final score. Moreover, they are often evaluated by applying personal criteria, such as by assigning greater significance to certain activities or topics that are not explicit or systematised and/or do not provide feedback or promote the achievement of learning, and usually take place without involving the student in the evaluation process (Stiggins 2002).

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Ethics statement

The study was undertaken in accordance with the British Educational Research Association's Ethical Guidelines for Educational Research

Disclosure statement

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