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Evaluation of local tolerance of a plant extract by the slug mucosal irritation (SMI) assay

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Abstract

This article describes the performance of a laboratory exercise, the Slug Mucosal Irritation (SMI) assay, carried out by third year undergraduate students, to investigate the local tolerance of an ethanolic plant extract. The plant extract, *Spilanthes acmella*, contains various bio-active compounds, such as the *N*-alkylamide spilanthol. After administration of the plant extract to the slugs, they were observed for possible discomfort and tissue damage. When slugs are exposed to a substance with irritant properties, the mucus production of the slugs will increase. Furthermore, slugs will release proteins, including enzymes, when tissue damage occurs. This laboratory experiment is a practically feasible *in vivo* test using slugs which are invertebrates that are not protected by the legislation on animal testing. Students were supervised by lab instructors who encouraged students to actively contribute in their groups, to think about the experimental design of the laboratory test, and to facilitate scientific discussions, but the majority of the ideas had to come from the students themselves. Hence, this biomedical *in vivo* experiment offered a great opportunity for students to learn to work in group, to critically interpret and report their results, to gain knowledge about the subject, and to communicate and discuss with other students as well as with the lab instructors. Furthermore, this experiment teaches students current toxicological methodologies encompassing principles and their application of biochemistry, analytical chemistry, toxicology, animal experimentation and data handling. This way of interdisciplinary teaching is especially important for last year undergraduate students, as this is a good preparation for the Masters dissertation. At the end of the laboratory exercise, students received a questionnaire and most of the students indicated that they gained valuable knowledge and skills. This laboratory exercise can be incorporated into chemical, biological, pharmaceutical, toxicological and medical disciplines.

Keywords

Model Systems; Toxicology; Undergraduate; Inquiry-Based; Student-Centered

Introduction

In the chemical, biochemical, pharmaceutical and medical field, it is important to evaluate the local tolerance and clinical discomfort of chemical, pharmaceutical and cosmetic formulations. Several studies have shown that the Slug Mucosal Irritation (SMI) assay is a useful tool for this evaluation. Moreover, slugs are invertebrate organisms that are not protected by the legislation on animal testing. Various protocols have been developed, differing in duration and number of contact periods, but all are based on the same principle: when a slug is exposed to a test substance, discomfort/irritation may be induced, which can be observed as an increase in mucus production. Discomfort is a mild form of irritation, experienced as stinging, itching and burning sensations. The outer single epithelial layer of the body wall of the slugs consists of epithelial and mucous gland cells overlying connective tissue. When tissue damage occurs, the mucosal cells will release proteins, including enzymes like lactate dehydrogenase (LDH), which can be quantified: LDH is an abundant cytosolic enzyme in cells and when tissues are damaged, LDH will be released.[1-7]
The SMI assay was performed by students of the third year of Bachelor of Pharmaceutical Sciences at Ghent University (Belgium). The students received a case-question about a plant-derived cosmetic product, which they had to solve by working cooperatively in small groups of 6 to 7 students. They were asked to evaluate the local tolerance of a plant extract (*Spilanthes acmella*, Asteracea), containing bio-active compounds such as the main lipidic compound, *i.e.* the *N*-alkylamide spilanthol.[8-11] Spilanthol is used in cosmetics due to its anti-wrinkle properties.[12] Various other functionalities are ascribed to spilanthol and has traditionally been used as a food spice.[13] The SMI assay is an efficient tool as it is a practically feasible *in vivo* study, *i.e.* undergraduate students quickly learn how to perform the assay. Students were supervised by lab instructors during the laboratory experiment who encouraged students' personal reflections and facilitated discussions, but the majority of the ideas had to come from the students themselves.

It is not only important to involve students in current toxicological methodologies to determine discomfort and local tissue damage of test substances, it is also important for them to practically apply a wide range of basic biomedical, biochemical, analytical and chemical scientific principles. Furthermore, in this way, students learn to write a protocol, gain practical experience with an animal assay, gain knowledge about the subject and learn to process data. They must also be able to scientifically discuss toxicity tests, taking into account ethical issues about animal tests and the 3 R principle (Reduction, Replacement and Refinement), legal regulatory requirements (*e.g.* difference between cosmetics and drugs) and developmental challenges of new, alternative toxicological test systems. The principles of Good Laboratory Practices (GLP) were also explained to the students. GLP is a quality system with a set of rules and criteria to ensure uniformity, reliability, reproducibility, consistency and quality of non-clinical health and environmental safety tests.[14] This exercise offers a great opportunity for students to learn to work in group, to interpret their results, to communicate and discuss with other students, as well as

with the lab instructors. For final year undergraduate students, this is a good preparation for the Masters dissertation.

EXPERIMENTAL SECTION

Scientific case question

At the start of the *Spilanthes acmella* project, students received the following scientific case: "A cosmeceutical company produces an ethanolic *Spilanthes acmella* extract as a topical anti-wrinkle product. Since the time the product has been on the market, there have been complaints of excessive skin discomfort. You are requested to investigate these complaints." The *Spilanthes acmella* plants were purchased from a local plant nursery. The plants were dried, and each group of students received either the flowers, the stems or the leaves. Subsequently, they had to make an ethanolic plant extract.

Slug Mucosal Irritation (SMI) laboratory exercise

The bioassay. To investigate if the *Spilanthes acmella* extract induces discomfort, a variant of the Stinging, Itching and Burning (SIB) protocol from the SMI assay was performed.[6] This was done in the course of two laboratory sessions taking four hours each. The first half-day was devoted to GLP, the introduction of the test, and the operational aspects (*e.g.* equipment qualification, sample preparation, labelling and weighing of the petri dishes). The test itself was executed on the second half-day. The amount of mucus produced by the slugs is a criterion for irritability/discomfort. The terrestrial slugs (*Arion lusitanicus*), weighing between 3 and 6 g, were collected in gardens and were subsequently kept in an acclimatized room (18-20°C).[6] The slugs were first carefully inspected for the absence of macroscopic injuries. They were placed in a plastic box containing a paper towel moistened with a phosphate buffered saline (PBS) solution and were kept at 18-20°C. During this two day pre-treatment, the body wall of the slugs was wetted once a day with 1 mL PBS.

Performance of the test. Several solutions containing different concentrations of the spilanthol extract (ranging from 0.2 to 10.0% with the negative control corresponding to 0%) were evaluated. The students had to determine if there was a relationship between the concentration of the extract and the slug mucus production. Since the *Spilanthes acmella* extract is not soluble in PBS, students were advised to add a solubilizer. Co-solvents (*e.g.* up to 5% ethanol) or surfactants (*e.g.* up to 2.5% Tween 20) can be used. Since these chemicals themselves are irritating to the slugs, the concentration is important as it will influence the results. Therefore, no more than 5% ethanol or 2.5% Tween 20 was used, as these concentrations do not irritate the slugs (unpublished data).

In order to ensure accurate and consistent performance, students had to qualify the micropipettes before preparing the dilution series. The use of proper controls is another essential aspect of scientific experiments to be grasped by students. Students had to figure it out themselves (literature search) which controls they had to use. Two controls should always be included: PBS

and a 1% w/V benzalkonium chloride (BAC) solution in PBS, both containing 2.5% Tween 20 or 5% ethanol, were used as a reference for a non-irritating (negative control) and strongly irritating/tissue damaging substance (positive control), respectively. Each prepared solution (*i.e.* negative and positive control and different concentrations of spilanthol extract) was added to three slugs (n=3). For every treatment, three slugs were placed individually in pre-weighed petri dishes. A Mettler Toledo Precision Balance PB153-L was used with a readability of 0.001 g. The initial body weight of the slugs was determined for each slug separately, by reweighing the petri dishes with the slugs and subtracting the weight of the empty petri dishes. Next, 100 µL of the test solution was added with a micropipette nearby the foot of the slug and left in contact for 15 minutes. The slugs were subsequently transferred into a second pre-weighed petri dish to determine the weight of the slugs again: subtracting the weight of the empty petri dish of the weight of the second petri dish containing the slug. In that way, the reduction in body weight was determined. The mucus production was calculated as follows: the weight of the petri dishes in which the slugs were exposed to the test solution was determined (weight of petri dish containing test solution and mucus – weight of empty petri dish containing test solution = weight of produced mucus of the slug). The mucus production was expressed as a percentage (w/w) of the initial body weight of the slug before applying the test substance. Subsequently, 1 mL of PBS was added to the slugs and left in contact for one hour. If tissue was damaged by the treatment, the slugs released proteins and LDH enzyme in the PBS solution, which were measured using appropriate procedures as described hereunder. After one hour, the slugs were removed and transferred into another fresh pre-weighed petri dish. The body weight of the slugs was determined again for each slug separately. The slugs were exposed for a second time to the test substances (100 µL) for another 15 minutes (second contact period). The slugs were subsequently transferred into a second, pre-weighed petri dish and the weight of the slugs was determined again. The amount of mucus produced was calculated again as previously described. To the slugs in the second petri dish, 1 mL of PBS was added with a contact period of one hour. Afterwards, the slugs were transferred into another fresh pre-weighed petri dish and the body weight was determined. The total mucus production was calculated by taking the sum of the mucus production of the two contact periods. Tissue damage is predicted by the mean LDH release and the mean protein release. The total LDH and protein content was calculated by taking the sum of the results of both PBS samples.

Protein quantification. After one hour of exposure to the PBS solution, the protein concentration of the solution was determined with a NanoOrange® protein quantification kit (Invitrogen™, Merelbeke, Belgium) and expressed as µg/mL per gram body weight (normalized for the body weight of the slug). Proteins in solution can be measured accurately at concentrations between 10 ng/mL and 10 µg/mL using the NanoOrange® reagent.[15] In aqueous solution, the NanoOrange® dye reagent is initially nonfluorescent, but upon interaction with proteins, it exhibits a significant fluorescent signal which is correlated with the protein concentration. Fluorescence was measured in a fluorometer (Wallac 1420 multilabel counter Victor 2, PerkinElmer, Turku, Finland) using excitation/emission wavelengths of 485/590 nm.

Bovine serum albumin (BSA) was used as a calibration reference. For the quantification of the protein content in the PBS samples, students had to create a calibration curve with BSA standard solutions. More detailed operational information is described in the Supplementary Information.

LDH enzyme activity. After one hour of exposure to the PBS solution, the LDH enzyme activity, expressed as U L^{-1} g^{-1} per gram body weight (*i.e.* activity concentration, normalized for the body weight of the slug), was determined using a commercial enzyme kit (DG 1340-UV, Sigma Diagnostics, Bornem, Belgium), based on the standard method recommended by the German Society for Clinical Chemistry.[16] The assay is based on the decrease in absorbance at 340 nm, during the conversion of pyruvate and nicotinamide adenine dinucleotide hydride (NADH) into lactate and nicotinamide adenine dinucleotide (NAD^+), as only NADH absorbs light at 340 nm and NAD^+ does not. More practical details can be found in the Supplementary Information.

Objectives of the test. Students designed the experiment and wrote the protocol as part of the laboratory exercise. They had to determine the extent of mucus production and calculate the protein content and the LDH activity. Finally, they were asked to assess their results by comparison with the criteria for the classification of discomfort (Table 1) and tissue damage (Figure 1). They had to critically interpret the dose response curves for the mucus production, the protein content and LDH activity. Afterwards, students had to report and present their results. To determine the impact of this teaching method on students' gained knowledge and understanding during the course, students received two questions, *i.e.* (1) you found the course intellectually challenging and stimulating, (2) the course gave you valuable knowledge and skills, *i.e.* a gain in your educational program. They could choose between five different answers: they could totally not agree, not agree, agree, totally agree with the proposition or have a neutral opinion.

Table 1. Cut-off values for classification of discomfort (based on standard protocol of the Stinging, Itching and Burning test).[6]

Total mucus production (%) (n=3)	**Discomfort**
≤ 3 %	No
< 3 and ≤ 8 %	Mild
> 8 and ≤ 15 %	Moderate
> 15 %	Severe

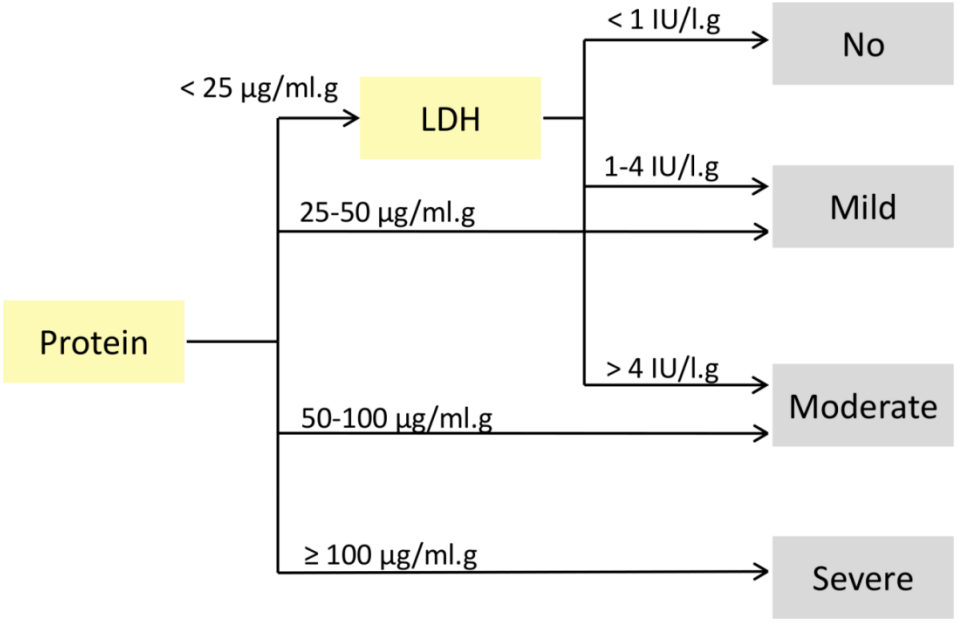

Figure 1. Prediction model for the interpretation of tissue damage (based on the protocol of the five-day local tolerance test).

Evaluation of the students

The lab instructor scored the students on a six point Likert scale, by evaluating how well students prepared the experiment, how well they understood the scientific and regulatory principles and how well they actively contributed in the execution and group discussion leading to results (0 = this person has done nearly nothing, 1 = the contribution of this person was minimal or just a little more than minimal, 2 = this contribution of this person could be better, 3 = this person contributed well, did her/his part, 4 = this person contributed more than well and 5 = this person has contributed excellent, there is almost nothing to criticize). There could only be given once a 5 and two times a 4. On the other hand, students had the opportunity to evaluate students from the same group, including themselves, according to their contribution in the group. The peer evaluation is a meaningful evaluation to evaluate the individual performance and working attitude in a group. The same six point Likert scale as described before was used for the peer review.

HAZARDS

During the first half-day of the SMI assay, a safety training was given to the students, including information concerning waste handling. They wore lab coats and safety goggles during the laboratory experiments. Furthermore, students used gloves in the laboratory.

RESULTS

Students succeeded in writing a protocol and successfully carried out the Slug Mucosal Irritation assay. During the laboratory exercise, the lab instructor had a scientific discussion with the students about the subject (*i.e.* alternative toxicological tests, legal regulatory requirements, ethical issues) and GLP aspects, which is important to obtain valid test results. Students were evaluated by the lab instructor on their degree of participation, using the six point Likert scale. After the data of the practical test were obtained, students calculated the results and discussed their findings in a report. Typical experimental results obtained by the students are presented in Table 2.

They compared their results of mucus production with the existing classification model for discomfort (three contact periods; Table 1). The more mucus the slugs produced, the more local discomfort was expected. It appears that some of the prepared *Spilanthes acmella* extract solutions caused a mild discomfort effect to the slugs. No clear concentration-dependent effect was observed. Furthermore, the LDH and protein contents were also compared with the prediction model for the interpretation of tissue damage. In Figure 1, limits are given which correspond to no, mild, moderate or severe tissue damage, respectively.[17] The non-LDH branches in the figure indicate the total protein content. According to the prediction model, below a total protein content of 25 µg/mL per gram body weight, to evaluate the tissue damage, the LDH activity must be taken into account. However, low protein levels and no LDH were found (*i.e.* below the detection limit) in PBS contacting slugs, to which the negative control and plant extracts were added. They were only present in PBS of the slugs which came in contact with the positive control, indicating the validity of the test method. To conclude, no tissue damage occurred, except for 5% SPE of group B and different discomfort effects were observed depending on the concentration and the plant extract. Students discussed the possible causes of the observed discomfort. Not only bio-active compounds, but also residual extraction solvents can cause irritation to the slugs. Students also realised that the biological variability of the results of *in vivo* experiments is higher than the analytical variability.

At the end of the exercise, students filled in the two questions they have received and the results are presented in Table 3.

Table 2. SMI-test results of the 3 groups with indication of the classification for discomfort and tissue damage.

Group Part of plant	Test substance	Total MP (%)	Classification Discomfort	Protein content (µg/mL.g)	LDH activity (U/L.g)	Classification Tissue damage
Group A	Negative control	0.6 ± 0.3	No	7 ± 3	-	No
Flower	Positive control	15.3 ± 1.9	Severe	68 ± 37	1.2 ± 0.5	Moderate
extract	SPE 0.20 %	2.7 ± 0.4	No	6 ± 3	-	No
	SPE 0.50 %	3.9 ± 0.6	Mild	8 ± 0	-	No
	SPE 0.56 %	4.2 ± 0.5	Mild	5 ± 1	-	No
	SPE 0.61 %	3.1 ± 0.3	Mild	8 ± 5	-	No
	SPE 0.67 %	3.5 ± 1.1	Mild	24 ± 26	-	No
Group B	Negative control	0.6 ± 0.6	No	17 ± 6	-	No
Leaf	Positive control	14.2 ± 1.5	Severe	101 ± 69	0.5 ± 0.4	Severe
extract	SPE 0.31 %	0.1 ± 1.0	No	11 ± 7	-	No
	SPE 0.63 %	0.3 ± 0.8	No	18 ± 5	-	No
	SPE 1.25 %	1.7 ± 1.9	No	17 ± 8	-	No
	SPE 2.50 %	2.8 ± 0.6	No	9 ± 3	-	No
	SPE 5.00 %	3.0 ± 0.5	Mild	41 ± 23	-	Mild
	SPE 10.00 %	3.6 ± 1.0	Mild	7 ± 1	-	No
Group C	Negative control	0.2 ± 0.4	No	14 ± 6	-	No
Stem	Positive control	11.0 ± 2.5	Moderate	95 ± 63	0.2 ± 0.3	Moderate
extract	SPE 0.16 %	0.8 ± 0.6	No	21 ± 12	-	No
	SPE 0.31 %	0.8 ± 0.3	No	16 ± 6	-	No
	SPE 0.63 %	1.1 ± 0.5	No	14 ± 7	-	No
	SPE 1.25 %	1.2 ± 0.5	No	14 ± 9	-	No
	SPE 2.50 %	1.7 ± 0.3	No	10 ± 11	-	No
	SPE 5.00 %	2.6 ± 0.2	No	5 ± 3	-	No

MP = mucus production; LDH = lactate dehydrogenase; Negative control = PBS (phosphate buffered saline) with 2.5% Tween 20; Positive control = benzalkonium chloride 1% with 2.5% Tween 20; SPE = spilanthol extract; Total MP, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 3 slugs; - = below the detection limit

Table 3: Learning objectives evaluation form.

Question	Totally not agree (%)	Not agree (%)	Neutral (%)	Agree (%)	Totally agree (%)	Not filled in (%)
1. You found the course intellectually challenging and stimulating.	0.68	6.16	30.82	56.85	4.79	0.68
2. The course gave you valuable knowledge and skills, *i.e.* a gain in your educational program.	0.00	8.22	23.97	60.96	6.85	0.00

For question 1, 57% agreed with the proposition and 5 % totally agreed with it; while 31% of the students had a neutral opinion. Hence, the majority of the students found that the course was intellectually challenging and stimulating. The percentages of the students that agreed (61%) and totally agreed (7%) with question 2 was even higher than for question 1. It can be concluded that most of the students indicated that they gained valuable knowledge and skills from this course.

At the end of the laboratory exercise, the students were scored, taking into account the peer evaluation and the points given by the lab instructor.

CONCLUSIONS

This article describes the integration of the Slug Mucosal Irritation assay in the curriculum of third year undergraduate students. By performing practical tests in small groups (6-7 students), students had to find an answer on a given scientific case about the local tolerance of the *Spilanthes acmella* extract. To resolve this case, they had to design the experiment, prepare the protocol and finally correctly perform the SMI assay, which is an *in vivo* toxicology test. At the end of the laboratory exercise, students were evaluated by the lab instructor, as well as by the other students of their group. Moreover, they were questioned about the learning objectives. The majority of the students gained valuable knowledge from this test. This laboratory experiment can be applied in chemical, biological, toxicological, pharmaceutical and medical advanced courses in biomedical curricula.

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