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Laboratory Experiment

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What Is the "Areca" in "Areca Nuts"? Extraction and Neuroactive Bioassay of Arecoline

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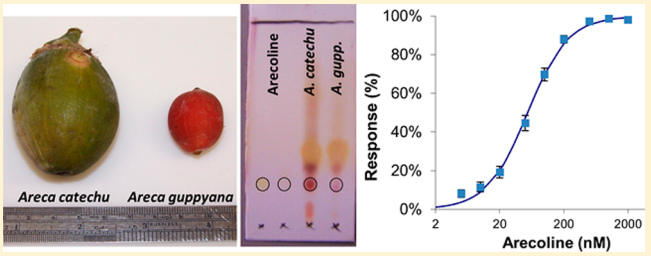
Supporting Information

ABSTRACT: A series of three practical sessions are designed to give students firsthand experience with the preparation of natural product extracts and assay using a live tissue preparation. Areca or betel nuts are the seeds from the fruit of the *Areca catechu* palm tree that is known to contain a number of pharmacologically active alkaloids. The principal of these is arecoline that makes up to 1% of the dry nut. Arecoline is a potent spasmogenic agent, causing smooth muscle contraction via muscarinic acetylcholine receptor (mAChR) activation. The first session involves the preparation of methanolic extracts from whole areca nuts and TLC for the qualitative identification of arecoline present in the extract. The second session utilizes the spasmodic effects of arecoline on smooth muscle to allow students to perform a live tissue bioassay using guinea-pig ileum. This response is subsequently blocked by the mAChR antagonist atropine to investigate the mechanism underlying the measured response. The final session gives students the opportunity to construct arecoline dose−response curves based on their experimentally derived data. From this curve and the obtained antagonist results, they are able to calculate an estimate of the arecoline content in the extracts they prepared and the original betel nut samples.



KEYWORDS: *Second-Year Undergraduate, Upper-Division Undergraduate, Hands-On Learning/Manipulatives, Biosynthesis, Chromatography, Medicinal Chemistry, Natural Products, Drugs/Pharmaceuticals*

BACKGROUND

Natural products have continued to provide a valuable source for chemical leads in the development of pharmaceuticals.[1] In fact, it is estimated that up to 70% of newly approved drugs in the last 30 years have been derived from or had some origin in natural products;[2,3] hence, it is imperative that natural products be fully represented in undergraduate pharmacology courses.[4−7] This series of practicals is designed to give students experience in natural product neuropharmacology via the extraction and bioassay of neuroactive compounds in the areca nut.

Areca nuts are the seeds from the fruit of the *Areca catechu* palm tree. They are often referred to as betel nuts as they are typically chewed with leaves from the betel plant. Betel nut chewing is a common practice throughout Asia,[8] and is the most frequently used drug in the world after tobacco, ethanol, and caffeine.[9]

The areca nut contains a number of pharmacologically active alkaloids, including arecoline, arecaidine, guvacoline, and guvacine.[9] Arecoline is the principle agent, found in up to 1% of the dry weight of the nut.[10,11] Arecoline is a cholinomimetic and has a number of structural similarities to acetylcholine (Figure 1), a major neurotransmitter involved in central and autonomic nervous system signaling.

Arecoline acts as muscarinic acetylcholine receptor (mAChR) agonist and is thought to be the primary active ingredient in areca nut, mediating a number of the central nervous system effects of the preparation. Arecoline itself has been trialled in various studies as a nootropic to mitigate cognitive decline in Alzheimer's disease,[12−16] although its purported carcinogenic effects[17−20] have limited its application in such areas.

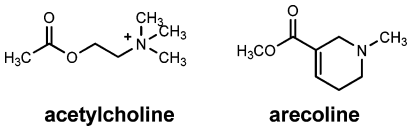


Figure 1. Structures of acetylcholine and arecoline.

Due to the spasmodic effects of arecoline on smooth muscle via mAChR activation at the neuromuscular junction of the parasympathetic autonomic nervous system, this series of experiments gives students a valuable opportunity to test the effects of arecoline firsthand using a live tissue preparation.

EXPERIMENTAL OVERVIEW

This series of three practical sessions is designed to give students firsthand experience with the preparation and biological assay of natural product extracts. The first session involves the preparation of methanolic extracts from whole areca nuts and TLC for the qualitative identification of arecoline present in the extract. The second session utilizes

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the spasmodic effects of arecoline on smooth muscle to allow students to perform a live tissue bioassay using guinea-pig ileum. This response is subsequently blocked by the mAChR antagonist atropine to investigate the mechanism underlying the measured response. The final session gives students the opportunity to construct arecoline dose−response curves based on their experimentally derived data. From this and the obtained antagonist results, they are able to calculate an estimate of the arecoline content in the extracts they prepared and the original betel nut samples. Institutional ethical approval for the use of animals in experimentation is required to conduct this practical.

EXPERIMENT

Session 1: Preparation of Areca Nut Extract

This session requires 3 h to complete and students work individually or in groups of up to four students. Students prepare methanolic extracts (50 mL) from fresh whole nuts (10−50 g total weight of husk and kernel) from a species of the Areca palm. Please note that for the reported experiments, methanol, which is classed as a grade two solvent according to ICH standards, is utilized. Ethanol, a class three solvent, may be substituted for methanol to lower risk of toxicity. Following filtration of the methanolic extract, students examine the extract for the presence of arecoline using TLC against arecoline standards (Merck silica gel 60 F_{254} in ethanol/*n*-butanol/acetic acid/water, 3:1:1:1 (v/v), visualizing with ninhydrin). For full details of this procedure, please consult the tutor guide in Supporting Information. The filtered extract is evaporated to dryness in vacuo to provide a final complex mixture constituting 2−5% of the total nut weight. Dry extracts are stable to cold storage for a week or more with exclusion of light and moisture. The session ends with a discussion of extract yield and the reliability of TLC identification of compounds (questions to guide discussion included in tutor guide (Supporting Information)).

Session 2: Bioassay of Areca Nut Extract

This session requires 3−4 h and is performed in groups of two to four students. Guinea pig ileum is used as a functional model of nervous system-effector activity, whereby receptors on the ileum produce an observable, robust response that is easy to replicate and to quantify.

Part A: Arecoline Dose Response Curve. For full details regarding the preparation of stock solutions and subsequent serial dilutions please consult the tutor guide (Supporting Information). Note that student values are to be recorded in worksheet 1, labeled "Arecoline Dose Response Data" of the excel spreadsheet provided in the Supporting Information. Using a 3 min dose cycle (Figure 2), starting at a low dose of arecoline (0.1 mL of 1 μM) and stepping through higher doses, students construct a full dose−response curve. Students apply increasing doses of arecoline until a maximal response is obtained. Demonstrators should ensure that students stick to all suggested dose cycles to ensure adequate tissue recovery time. This reduces the occurrence of tissue fatigue and desensitization. From their raw data values, students identify maximal effective concentration (EC_{max}) and 40−60% (EC_{40-60}) arecoline doses and their associated responses measured as contractile response in arbitrary units.

Part B: Areca Extract Responses. Using the same 3 min dose cycle, students establish a dose of areca extract with an equivalent EC_{40-60} response. Students begin with a low dose

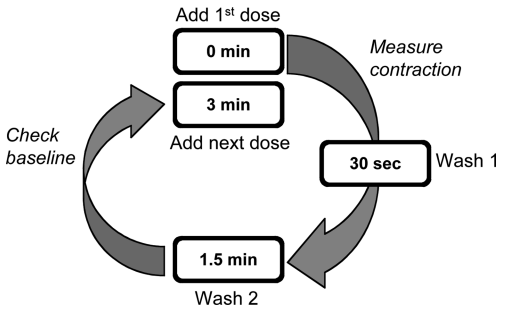

Figure 2. Agonist dose cycle.

(0.2 mL of the 1/10000 dilution), increasing in log dose steps until the first response is observed, before a more metered investigation of appropriate doses to attain the desired level of response. Student values are to be recorded in worksheet 3, labeled "Areca Extract Results" of the excel spreadsheet provided in the Supporting Information.

Part C: Antagonist Experiments. The selective, competitive mAChR antagonist atropine is co-applied with the extract to ascertain the proportion of extract activity resultant from mAChR activity. An antagonist dose-cycle is entered (Figure 3) beginning with a 0.2 mL of 1 μM dose atropine and

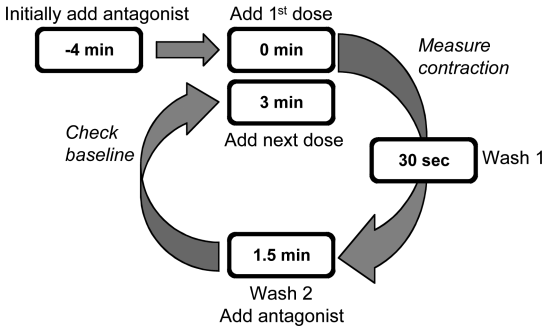

Figure 3. Antagonist dose cycle.

equilibration for 4 min to allow sufficient receptor occupation before the addition of an EC_{40-60} dose of arecoline. This concentration of atropine typically completely blocks the arecoline response, but students may require higher doses. Using this dose of atropine, students repeat the antagonist dose-cycle, this time adding the EC_{40-60} extract dose. Amplitude of response alone and in the presence of atropine are recorded for both arecoline and extract and entered into worksheet 4, labeled "Antagonist Experiments" of the excel spreadsheet provided in Supporting Information. The session ends with a discussion regarding the mechanism of action behind measured tissue activation and the role of the antagonist experiments in the exercise.

Session 3: Data Analysis Workshop

This session takes 2 h. Students analyze the class results to construct arecoline dose−response curves, calculate extract response due to mAChR activation, and derive the content of arecoline present in extracts and the original areca nut. A model Microsoft Excel spreadsheet and step-by-step instructions have been included in the Supporting Information to guide students and tutors through the required calculations.

Part A: Calculation of Arecoline Dose Response Curve. Using data from Session 2, and working within worksheet 2, "Class Arecoline DR Curve", of the excel

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spreadsheet provided in Supporting Information, a dose–response curve relationship is generated according to the Hill equation.

$$\frac{R}{R_m} = \frac{1}{1 + \left(\frac{EC_{50}}{[D]}\right)^{n_H}}$$

whereby

R = response to a given dose (D)
R_m = maximum response
EC_{50} = the effective concentration to elicit a response that is 50% of the maximum
n_H = Hill coefficient.

Students utilize their experimental data and the inbuilt solver function of Microsoft Excel to calculate the EC_{50} and n_H values that most closely approximate this relationship. Directions for these calculations are in the tutor guide (Supporting Information).

Part B: Calculation of the Areca Extract Response due to mAChR Activation. Calculations for this task are performed in worksheet 5, labeled "Raw Data Summary + Calculations" of the excel spreadsheet provided in the Supporting Information. Students identify the proportion of the response due to mAChR activation from their test of the $EC_{40–60}$ dose of extract in the presence of a competitive mAChR selective antagonist atropine. Any residual response is due to other receptor classes.

To calculate the equivalent arecoline doses, students rearrange the Hill equation to the following form:

$$[D] = \frac{EC_{50}}{\left(\left(\frac{1}{R_r}\right) - 1\right)^{1/n_H}}$$

Solving for the response of the extract due to arecoline (R_r = measured response − residual response following atropine treatment), and the derived EC_{50} and n_H values from Part A, gives the dose of arecoline present in the extract (D). From this, students derive a molar concentration of arecoline present in the dried extract. Similarly, considering the yield of extract and weight of areca nuts, they derive the arecoline content (% w/w) of the sample, suitable for comparison with literature values.

HAZARDS

Potentially hazardous and flammable reagents are used during this practical. Methanol is flammable and toxic if in contact with the skin, by inhalation and if ingested. It is also irritating to the eyes, skin and mucous membranes and may cause eye damage through prolonged or acute exposure. Do not allow methanol to contact the skin, eyes, and clothing. The MSDS (Material Safety Data Sheet) sheet for methanol stipulates the use of safety glasses and gloves and the provision of good ventilation. *n*-Butanol is flammable, toxic if swallowed and can cause skin irritation, eye damage, respiratory irritation, and drowsiness or dizziness. Acetic acid is flammable and can cause severe skin burns and eye damage. Ethanol is highly flammable and can cause serious eye irritation. Ninhydrin is harmful if swallowed and can cause skin, eye, and respiratory irritation. The areca nuts, their corresponding extract, arecoline and atropine either are, or contain, neurologically active species. Care must be taken when handling these substances. Contact with skin should be avoided and suitable personal protective equipment worn.

Guinea pigs are docile rodents and rarely inflict injuries; however, individuals should be trained in the appropriate handling techniques to minimize risk. Individuals can commonly develop asthma and allergy responses to guinea pigs. Dust masks, gloves and long sleeved apparel should be worn when handling guinea pigs. Guinea pigs are also associated with a low risk of biological infection. This risk can be minimized through the use of appropriate personal protective equipment and ensuring that hands are washed after coming in contact with rodent saliva, urine, blood, feces, and/or bedding material.

RESULTS AND DISCUSSION

These practical sessions have been run each year for 3 years in an upper-division undergraduate pharmacology course entitled "Neuropharmacology" across a total cohort of over 300 students. The success of this series of practicals is in part determined by how well students are able to break down the nut during Session 1 as it helps to maximize the extraction of active alkaloids for later bioassay measurements. The presence of arecoline in the areca extracts is ascertained using TLC. TLC is a routine qualitative method used by chemists as it can quickly and fairly accurately identify the presence of compounds in a mixture.[21] Our investigations found ninhydrin provided the most robust visualization method; however, groups may also want to examine other stains such as Dragendorff's reagent or potassium iodoplatinate that are alkaloid specific.[22]

Arecoline is commonly found in most species of the *Areca* family, but our work focused on both *Areca catechu* and *Areca guppyana*, both found to provide sufficient concentrations of arecoline for subsequent testing. If it is possible to source various species of nut, this also gives students the ability to compare arecoline content across species in their final session. Students utilized the in-built Solver function of Microsoft Excel to calculate derived EC_{50} and n_H values for tissue response to increasing arecoline concentrations. Solver allowed students to find an optimal (minimum or maximum) value for a given formula (objective cell), subject to designated limits. This is a powerful and easily accessible function that, if students are able to master, may find use in a wide array of analytical, biological and chemical protocols. Derived values were typically 1.1–1.7 for n_H and 30–60 nM for EC_{50}. As a guide, the derived arecoline dose response curve generated from student data obtained over a three year period is shown in Figure 4. This exercise gave students experience with a key skill in pharmacology, that of calculating corresponding dose–response relationships from experimental data, all achieved using generalized and commonly available software.

A spreadsheet was provided that guides students, step-by-step through the calculations required to derive final % w/w values for arecoline content from measured extract responses. Averaged student results for these are in Table 1. Demonstrators should note, however, that these values relate to fresh nut samples and will not be comparable with literature values for dry nuts. Full details of these calculations are given in the tutor guide (Supporting Information).

This method is robust and reproducible, with students typically obtaining comparable results within a given cohort for the arecoline dose response curve and the arecoline content of the areca nuts. Student data obtained consistently showed

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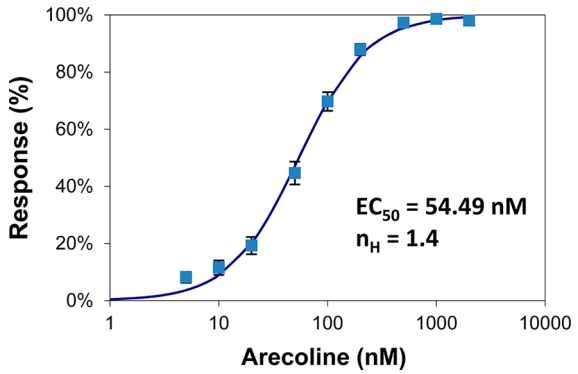

Figure 4. Arecoline dose response curve based on student data obtained across a three year period. Data are mean ± SEM (n = 5–28). Curve was generated in Microsoft excel using the spreadsheet provided in the Supporting Information.

Table 1. Student-Derived Arecoline Content of Areca Nut Species[a]

Areca Nut Species	Arecoline Concentration in Dry Extract (% w/w)	Arecoline Concentration in Fresh Nut (% w/w)
Areca catechu	$6.7 \times 10^{-2} \pm 6.4 \times 10^{-3}$	$3.0 \times 10^{-3} \pm 2.8 \times 10^{-4}$
Areca guppyana	$1.0 \times 10^{-2} \pm 1.3 \times 10^{-3}$	$3.6 \times 10^{-4} \pm 4.4 \times 10^{-5}$

[a]Data calculated are mean ± SEM (n = 10–18).

species differences in arecoline content, with the *A. catechu* nuts giving concentrations some 8-fold higher than that of the *A. guppyana*.

SUMMARY

The principle pedagogic goal of the experiment was to give students firsthand experience with the preparation and biological assay of natural product extracts, allowing them to recognize that plants contain neuroactive compounds that may be of interest in drug discovery. This goal was met through the three-week experiment that covered the preparation of methanolic extracts from whole areca nuts (week 1, 3 h), a live tissue bioassay on guinea-pig ileum (week 2, 3–4 h), and data manipulation of obtained results to derive estimates of arecoline concentration in the extracts (week 3, 2 h). The achievement of this goal was evidenced by strong student performance in the associated practical report (for further details see tutor guide in the Supporting Information) and the final examination in the sections on natural products as leads for neuroactive drug discovery.

These practical sessions have been run in an advanced undergraduate pharmacology course entitled "Neuropharmacology" for three years, across a total cohort of over 300 students. We have found this method to be robust and reproducible, with students typically obtaining similar results within a given cohort of the areca nuts. Some variance can be expected between cohorts, however, as there can be significant seasonal variance with areca nuts. Demonstrators should also note that difficulties may occasionally arise due to the natural variability of live tissue preparations.

This series is a unique and comprehensive introduction into natural products as sources for biologically active compounds. While a number of previously reported undergraduate laboratory experiments may deal with a subset of aspects relating to this area, such as the extraction of compounds[6,7,23−27] or bioassays to determine activity,[28−30] to the best of the authors' knowledge, there have been no reports of a single series that effectively covers both of these aspects in an integrated fashion. Of particular note is that this series gives students a valuable opportunity to see firsthand the spasmodic effect of arecoline on smooth muscle in an in vivo bioassay. When students first observe the action of a drug on live tissue, it can often be one of the most engaging moments students experience in Pharmacology. The series of experiments described here fulfill many learning outcomes in Pharmacology, which is an intrinsically interdisciplinary field. These experiments could alternatively provide a valuable interdisciplinary learning activity, for example, where students may isolate the alkaloid in an organic chemistry laboratory before performing the bioassay in a vertebrate physiology laboratory.

Overall, this was essential training for students and provided "hands on experience" for all students that helped to reinforce many basic pharmacological principles. That said, the authors acknowledge that the use of live animals for laboratory experiments can be costly and time-consuming to establish. Significant information has been provided in the tutor guide (Supporting Information) to assist with the preparation of ethics applications, providing clear justification for the use of the animals and evidence that the impact on animal well-being has been considered.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00312.

Tutor materials (PDF, DOCX)
Student handout (PDF, DOCX)
Bioassay with collated student data (XLSX)
Blank bioassay spreadsheet (XLSX)

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Notes

The authors declare no competing financial interest.

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