Assessing various antioxidant properties conditions to extract from *Valeriana wallichii* using colorimetric assays

John Bearden[1], Valeria Rivera[2], Alyssa Terrazas[3], Michael Hwang[4], Dr. Rachid Skouta[4]
[1]Chapin High School, [2]Andress High School, [3]Irvin High School, [4]The University of Texas at El Paso

Research Significance

Several diseases and cancers have been attributed to an imbalance of free radicals in our bodies. Antioxidants are able to quench free radicals within our bodies. This makes antioxidants a potential source of treatment and/or prevention for neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Plants are a great source of natural antioxidants that can be extracted using various solvents, temperatures, and times. This research experiment is being conducted primarily to determine which conditions would be most effective at extracting antioxidant activity from *Valeriana wallichii*. Once the antioxidant properties of *Valeriana wallichii* have been evaluated, potential future directions are to test the extracts in various cell lines to see its efficacy in the protection of healthy cells or destruction of cancer cells.

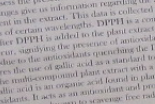
Research Question

What are the optimum conditions that will extract the most antioxidant activity from *Valeriana wallichii*?

Introduction

Free radicals are atoms or groups of atoms that have an odd number of electrons. In order for them to be neutral, free radicals take the electrons from other atoms and molecules in our body such as lipids, DNA and Proteins. When free radicals are in excess, they can cause damage to many cells in our body, generating a variety of neurological disorders, cancers, and diseases. On the other hand, damage due to free radicals and other surroundings in the environment like cigarette smoke or pollutants can be prevented by antioxidants. Antioxidants are molecules that can prevent the oxidation of other molecules, which are ideal for counteracting the damage caused by free radicals. The antioxidants in our bodies are very beneficial when it comes to free radicals due to the fact that they bond and stabilize the free radicals. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and Polyphenol assay are some of the simplest colorimetric assays which give us information about the activity of antioxidants and number of polyphenols present in the plant extract.

Colorimetric assays are used to assess the presence and concentration of certain compounds in solutions. Certain color changes give us information regarding the antioxidant activity and polyphenol quantity present in the extract. This data is collected using a plate reader that measures the absorptions of certain wavelengths. DPPH is a compound that is composed of free radicals. After DPPH is added to the plant extract, a color change from purple to yellow will occur, signifying the presence of antioxidants in the plant extract. The color change is due to the antioxidants quenching the DPPH free radicals. The polyphenol assay requires the use of gallic acid as a standard to compare the quantity of polyphenols to the antioxidant plant extract with a known and quantifiable polyphenol. Gallic acid is an organic acid found in plants and many foods that contain powerful antioxidants. It acts as an antioxidant and protects cells against any oxidative damage. Gallic acid has been known to scavenge free radicals such as hydrogen peroxide.



Abstract

Herbal medicines have become increasingly important to the field of medicinal chemistry due to their low toxicity, easy availability, noticeable bioactivity, and versatile effectiveness. Antioxidant properties are of particular interest for their role in protecting free radicals. Free radicals or reactive oxygen species (ROS) have been connected to neurodegenerative disorders, viral infections, autoimmune pathologies along with diseases, endogenous chemicals, and particularly stress hormones. Valeriana wallichii has been used for centuries as a mild sedative and a treatment for anxiety disorders. The alkaloid, Chatamine and Valerianin as well as other compounds like the antioxidant flavonoid, Hesperidin. This study will seek to further investigate the antioxidant properties of valerian extracts as well as to find the optimal conditions for antioxidant extraction.

Water, ethanol, and a water/ethanol mixture will be used as solvents under different temperatures and times. One at room temperature, 7 days at room temperature, 4 hours @ 40 degrees Celsius and then 24 hours at room temperature, 4 hours @ 60 degrees Celsius, and a microwave extraction. It is hypothesized that extended times increase the chances for compound extraction, then extracting Valerian for 48 hours at 90 degrees Celsius should provide the best conditions for extracting the antioxidant properties. Using water as a solvent should be more effective than ethanol because there are several water soluble antioxidants and it is safer for biological use. Even though the antioxidant activity will be determined by the DPPH assay and polyphenol assay will be utilized to analyze and compare the antioxidant activity as well as polyphenol content of the extracts.

Methods

Extraction

1. Gather all the materials and make sure they are clean.
2. Measure 150 mL of distilled water, 150 mL of ethanol, and a mixture of 90 mL of ethanol and 60 mL of distilled water in three separate graduated cylinders. Measure 25 grams of *Valeriana wallichii* on a digital scale at least three times.
3. Distribute the ethanol, distilled water, and ethanol/distilled water mixture solvents into three separate round bottom flasks. Add 25 grams of *Valeriana wallichii* into each flask.
4. Turn on the hot plate with the oil bath and heat it at 90 degrees Celsius (variable).
5. Place the three flasks onto three different hot plates and attach a clamp to each flask.
6. Clamp the reflux condenser to the roundbottom flask.
7. Turn on the water system to let water flow through the reflux condenser.
8. Clean all of the used materials and get rid of any unnecessary materials/substances.

Filtration

1. Gather all the materials and make sure they are clean.
2. Attach two sheets of filter paper into a small filter funnel and pour the *Valeriana wallichii* extract to collect the filtrate. Do this twice.
3. Connect the vacuum hose to a flask and attach a stone filter.
4. Pour the celite into the stone filter and pour in methanol to ensure that the filter is clean. Turn on the vacuum.
5. Turn off the vacuum and detach the tube connected to the vacuum.
6. Dispose the methanol into an organic waste bin.
7. Attach the round bottom flask to the clamp and re-attach the stone filter with celite.
8. Pour the *Valeriana wallichii* solvent into the flask.
9. Repeat steps using another flask.
10. Let the filtrate air-dry to remove the solvent.
11. Clean up the lab area and all of the used materials. Discard of any other substances.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay:

Preparing DPPH 1X stock

1. Measure 5.9 mg of DPPH and so that we are going to add 10 ml of methanol, which should provide us with 20X solution.
2. We are going to take away 1 ml from the 20X and going to add it to 20 ml of methanol to make a 1X solutions (50 uM).

Preparing *Valeriana wallichii* for assay

1. Measure out 0.1 mg of *Valeriana wallichii* from each extract. Adding 1,000 ul of DMSO. Mix the two very well using a sonicator.

Preparing the assay plate

1. Make a blank column by adding 200 ul of methanol.
2. Make a standard by adding 100 ul of methanol to entire column.
3. Create a negative control column by adding 100 ul of methanol and 10 ul of DMSO to the first well (B4).
4. Create a positive control column by adding Vitamin E to the first one (B5).

4. Next we are going to prepare columns for each plant extract and each solvent is going to get its own trial.
5. To all of the empty wells we are going to add 100 ul of methanol.
6. For all of the columns, excluding the blank and standard, we are going to do a serial dilution.
7. We are going to cover each plate and prepare for readings.
8. We are going to read the absorbance at 517 nm using a TECAN plate reader at 10, 20 and 30 minutes.
9. The experiment will be repeated three times.
10. Create graphs and tables using excel.
11. The radical scavenging effect will be calculated using the formula below:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A0 is the absorbance of the methanol control and A1 is the absorbance of the valerian extract.

Polyphenol Assay

Preparing gallic acid stock solution (5 mg/ml)

1. We started by measuring 100 mg of gallic acid and to it we added 20 ml of Methanol.
2. We gathered eight separate containers and in each one we added 10 ml of Methanol. In each one we add different amounts from the gallic acid stock. We added .50, 100, 150, 200, 250, 300, 350, 400 µl into each container.
3. From each container we are going to take 100 µl into a microcentrifuge tube.

Preparing *Valeriana wallichii* assay tube

1. Calculate measurements of *Valeriana wallichii* to test the same concentrations used in the DPPH free radical assay.

Preparing Folin-Ciocalteu solution

1. Measure 200 ul of Folin-Ciocalteu Reagent (FCR) and add it to 1,800 ul of water. Then mix the two solutions.
2. From the FCR, take away 200 ul and add it to the gallic acid standards and *Valeriana wallichii*.
3. Cover the microcentrifuge tubes, and incubate it for 30 minutes at room temperature.

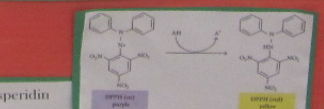
Preparing sodium bicarbonate solution (700 mM)

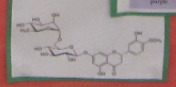
1. We measured 1,234.9 mg of sodium bicarbonate and add 21 ml of water. Shake the container to mix well.
2. From the mixture take away 800 ul and add it to the eight gallic acid standards and the *Valeriana wallichii*. Cover the container and incubate it for two hours at room temperature.

Preparing and reading the assay plate

1. Get a clean 96-well plate.
2. Create a blank by adding 250 ul into the first 3 wells.
3. From each of the assay tubes take away 250 ul and add it to the plate. Repeat this step 3 times for each assay tube.
4. Read the plate at 755 nm using a Tecan microplate absorbance reader.
5. Save and record the absorbance value.
6. Create gallic acid standard curve.
7. Calculate the averages and the standard deviations.
8. Create a gallic acid standard curve using excel.
9. Find the intercept and the slope of the curve.
10. Calculate the phenol concentration of *Valeriana wallichii* using the formula below:

$$\text{Phenol Concentration } \left(\frac{mg}{L}\right) = \frac{(\text{DPPH sample} - \text{DPPH blank}) - (\text{intercept of standard curve})}{\text{Slope of standard curve}}$$





Hypothesis

If higher temperatures and extended times increase the chances for compound extraction, then extracting Valerian for 48 hours at 90 degrees Celsius should provide the best conditions for extracting its antioxidant properties. Using water as a solvent should be more effective than ethanol because there are several water soluble antioxidants and it is safer for biological use.

Data That Will Be Obtained

The type of data that will be obtained during this experiment will be the antioxidant activity from *Valeriana wallichii* using different extraction conditions. The DPPH assay will determine the antioxidant activity and the polyphenol assay will determine the number of polyphenols in the extractions. These two assays will give us a better idea of the antioxidant activity and polyphenol content within the extracts by analyzing the absorbance readings for the two colorimetric assays. This information will allow us to decide on the optimal conditions for obtaining the most antioxidant activity from *Valeriana wallichii*.

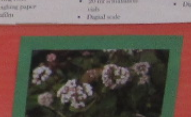
Materials

Extraction
- Dried root from *Valeriana wallichii*
- Distilled water
- Pure ethanol
- Distilled water/pure ethanol mixture
- Graduated cylinder
- Round bottom flask/Erlenmeyer flask
- Digital scale
- Hot plate
- Reflux condenser
- Spatula/stirrer
- Thermometer
- Stirring rods
- Weighing paper
- Parafilm

Filtration
- Funnel
- Filter Funnel
- Filter Paper
- Vacuum hose
- Vacuum
- Methanol
- Celite

DPPH Assay
- Overall plate
- DPPH
- Solvents: methanol and DMSO
- Controls: Vitamin E
- Pipette tips
- 20 ml scintillation vials
- Digital scale

Polyphenol Assay
- Overall plate
- Gallic Acid
- Sodium Bicarbonate
- Folin-Ciocalteu reagent (FCR)
- Microcentrifuge tubes
- Pipette tips
- DI water
- Methanol
- Scissors
- Foil
- 20 ml scintillation vials
- Graduated cylinder
- Digital scale



Acknowledgements

We would like to acknowledge our families, friends, and peers for supporting us through this process, as well as our teachers for informing us about this program. We would also like to thank our head scientist Dr. Rachid Skouta, program director Dr. Pei-Ling Hsu, and all of our RAs: Michael, Mary, Kassandra, Katherine, and Mariana for helping us with anything we needed and guiding us through this experience! We would also like to thank the National Science Foundation for providing the funding for this program.

* This project is sponsored by National Science Foundation, No. DRL-1322600

Resources

Ahmad, S., Mustafa, G., Afreen, R., Khurshid, A., Ahmad, N., Rahman, K., Ahmad, S., Kamal, M., Rahman, N., & Yasmeen, K. (2012). Valeriana wallichii: Phytochemistry and biological activities. Journal of Pharmacy Research, 5(1), 1-4.

Bajpai, V. K., Sharma, A., & Baek, K. H. (2013). Antioxidant and antimicrobial activities of essential oil and extracts of Valeriana wallichii rhizomes after hydrodistillation and storage. Food Control, 32(2), 582-589. doi:10.1016/j.foodcont.2013.01.028

Bajpai, V. K., Rahman, A., Dung, N. T., Huh, M. K., Lee, J., Choi, U. K., Na, M., & Kang, S. C. (2007). Comparative antimicrobial, antioxidant and cytotoxic activities of the essential oil and various extracts of Valeriana wallichii DC. Journal of Essential Oil Research, 19(6), 553-559. doi:10.1080/10412905.2007.9699317

Bajpai, V. K., Rahman, A., Dung, N. T., Huh, M. K., Lee, J., Choi, U. K., Na, M., & Kang, S. C. (2007). Comparative antimicrobial, antioxidant and cytotoxic activities of the essential oil and various extracts of Valeriana wallichii DC. Journal of Essential Oil Research, 19(6), 553-559. doi:10.1080/10412905.2007.9699317

Bajpai, V. K., Sharma, A., & Baek, K. H. (2013). Antioxidant and antimicrobial activities of essential oil and extracts of Valeriana wallichii rhizomes after hydrodistillation and storage. Food Control, 32(2), 582-589. doi:10.1016/j.foodcont.2013.01.028

Bajpai, V. K., Rahman, A., Dung, N. T., Huh, M. K., Lee, J., Choi, U. K., Na, M., & Kang, S. C. (2007). Comparative antimicrobial, antioxidant and cytotoxic activities of the essential oil and various extracts of Valeriana wallichii DC. Journal of Essential Oil Research, 19(6), 553-559. doi:10.1080/10412905.2007.9699317