Establishing Tissue Culture of King of Bitters (Andrographis paniculata) and Comparison of Callus Versus Plant Neoandrographolide Production

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Abstract

Neoandrographolide is a secondary compound endemic to Andrographis paniculata (King of Bitters) and gives the plant its anti-inflammatory property. Because of the widespread use of neoandrographolide and the low seed germination rate of Andrographis paniculata, creating a callus tissue culture, from which to extract neoandrographolide, has the potential to be beneficial to pharmaceutical companies. Callus was produced from King of Bitters plant tissue using the Mineo protocol for tissue extraction and the Murashige protocol for the callus media and incubation. HPLC-CAD analysis was done on a liquid extract of callus (1 g dried callus to 50 mL methanol) and leaf tissue (1 g dried leaves to 50 mL methanol) using the Li protocol. No detectable neoandrographolide content was found in the callus, but neoandrographolide was found in the plant leaf tissues. To facilitate neoandrographolide production in callus, elicitors, genetic manipulation, metabolic engineering, or the use of symbionts may be viable options.

Introduction

The King of Bitters (*Andrographis paniculata*) is an herbaceous plant in the family Acanthaceae. An herb is a plant that is used for its medicinal properties, scent, or flavor. This plant is cultivated in southern Asia and has been used for centuries in Asian and European folklore medicine as an herbal supplement and remedy to several ailments.[6]

This plant, named for its extremely bitter flavor, is used as a bitter tonic in traditionally known health care systems of many Asian countries.[14] In Chinese traditional medicine, *Andrographis paniculata* is used to rid the body of toxins and break fevers. In Scandinavian countries, this herb is used to treat the common cold. In Thailand, this plant was even included in "The National List of Essential Drugs A.D. 1999" because it has many medical benefits, such as anti-viral, anti-inflammatory, anti-diarrheal, anti-malarial, anti-cancer, hepatoprotective, and is immunostimulatory.[13]

Within the last few decades, research on *Andrographis paniculata* has become more abundant. Many studies and clinical trials on the medicinal and therapeutic properties of King of Bitters have also surfaced.[14] Studies have shown that the leaves of the mature plant contain andrographolide and neoandrographolide (Figure 1), which have been reported to possess immune-stimulant, anti-pyretic, anti-inflammatory, and anti-diarrheal properties.[4]

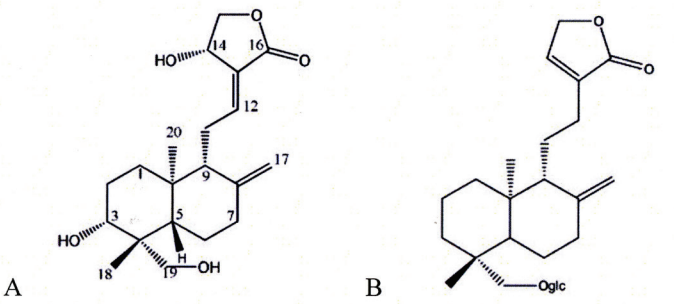

Figure 1: Structure of (A) neoandrographolide and (B) andrographolide.

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Under the humid tropical conditions of Asia, *Andrographis paniculata* normally reaches maturation 90-120 days after germination. A major issue in growing this plant is the poor rate of seed germination. The King of Bitters seed germination percentage rate is relatively low compared to many other plants. The rate of germination can be as low as 20%. Scientists believe that this is due to the difficulty of breaking this plant's dormancy due to its seed coat.[4]

Today, King of Bitters plants are harvested for their leaves and sold as an herbal medicine.[2] With the seed's difficulty to germinate, finding a faster, easier way to get the medicinal compounds from the plant would be valuable. A possible solution would use plant tissue culture to create *Andrographis paniculata* callus. Callus is a fast-growing mass of undifferentiated cells that a plant grows to defend itself. These cells can be used to create medicines or new plantlets more quickly and efficiently than from naturally growing a King of Bitters plant from seed to maturity.[15] Callus has been used with various plants with germination difficulties and has been found to be a much easier tissue from which to extract compounds. Therefore, establishing tissue culture callus for this plant could be beneficial. This can be done by taking cuttings from plants grown in the greenhouse and putting them on media containing Murashige and Skoog (M.S.) salt mixture, agar, sugar, auxin, and cytokinin. This causes the plant tissue to create callus.[12]

The medicinal compound of interest in this experiment is neoandrographolide. This compound is found solely in *Andrographis paniculata* and it contributes to the plant's anti-inflammatory properties.[10] Neoandrographolide inhibits nitric oxide and tumor necrosis factor-alpha production in lipopolysaccharide-induced macrophages, which leads researchers to believe the compound is the major contributing component of the anti-inflamitory property of *Andrographis paniculata*. This makes neoandrographolide a potential candidate for clinical trial.[10]

In this experiment, neoandrographolide content was compared between the leaves and callus. This was done by using HPLC-CAD analysis. The amount of neoandrographolide produced by callus and leaves were compared in order to understand the relative quantity of callus compound production. Andrographolide content was also observed for comparison with other studies.[9] These data will

determine if pursuing tissue culture for the King of Bitters would be efficient for pharmaceutical companies to produce neoandrographolide.

Methods

Seed Germination and Plant Maintenance

In order to break seed dormancy, the King of Bitters seeds (sourced from stock plant in Department of Biology greenhouse) were given three different treatments. Seeds were either soaked for 5 minutes in 50°C water,[7] soaked for 10 minutes in 2% KCl solution, or scarified with sand paper 5 times.[11] The seeds were then planted in 15 cm pots with commercial potting soil and put under a 24-hour misting system in the MTSU Biology greenhouse. Scarification gave the best results. Once plantlets arose, they were removed from the misting system. Plantlets were watered as needed and fertilized every two weeks. These plants required minimal care for growth.[7]

Media Preparation

Media were prepared using the protocol of Murashige.[12] This medium included agar, M.S. salts-5501, hormones (Naphthaleneacetic acid and Benzylaminopurine), and 3% sucrose. All media ingredients were obtained from Phytotech Laboratories. These ingredients are formulated to cause callus growth. Seventeen grams of M.S. salts (M5501) were added to a flask, which was filled to 400 mL with deionized water. A stir bar was added to mix the solution until the salt was dissolved. During this time, 750 **µL** of benzylaminopurine (BAP) hormone and 500 **µL** of naphthaleneacetic acid (NAA) hormone were added to the solution, along with 5 mL of casein. The flask was then filled with deionized water to total 500 mL and autoclaved. At the same time, 4.5 g of agar was autoclaved in a separate 1000 mL flask. Once autoclaved, the solution was added to the agar, mixed together, and poured into approximately 20 sterile petri plates (100 mm x 15 mm). These plates were then sealed with parafilm.

Callus Production

Cuttings of the leaves and stems were taken from the King of Bitters plants and sterilized using deionized water, 70% isopropyl alcohol, 0.2% plant tissue culture contamination control (PTC3) antibiotic, 2.5% bleach, and Dawn dish detergent. The tissues were rinsed with the deionized water, then washed in the 70% alcohol

for approximately 30 seconds. Next, the tissues were washed for 10 minutes in a mixture of the detergent, antibiotic, and bleach. Finally, the tissues were rinsed with sterile deionized water. Following the protocol of Mineo,[11] sterile scalpel and forceps were used to make small cuttings from the stem and leaves of the plant. Cuts were made to maximize the amount of internal tissue exposed. Sterile cuttings were placed on the media and plates were incubated at 25°C.

Leaf and Callus Drying

Calli were removed from media, put into empty petri dishes, and left partially open in a hood until the tissue was completely dried (for *Andrographis paniculata* callus, approximately 24 hours). The plant leaves were dried using a conventional leaf dryer for approximately 2 weeks.

Compound Analysis

The andrographolide and neoandrographolide analysis was carried out according to the protocol used by Li.[9] Callus and leaf tissues (1 g) were extracted using 50 mL methanol. The mixture was sonicated at 60% for 1 minute to lyse the cells and allow the compounds to dissolve in the methanol. The samples were then put on a shaker for 24 hours followed by filtration using a Buchner Funnel. The methanolic extracts were then concentrated to a final volume of 5 mL using a rotary evaporator (Büchi Rotavapor R-200) at 100 mbar in a 45°C water bath. The extracts were then further filtered using a 0.5 micron filter to remove particulates.

Standards for andrographolide and neoandrographolide were obtained from Sigma Aldrich in 5 mg quantities at 99.09% purity. Stock solutions were made at a concentration of 5 mg/mL in methanol. The authentic standards (diluted to 1 mg/mL) were analyzed on a high-performance liquid chromatography-charged aerosol detector (HPLC-CAD) system to generate reference chromatograms. Identification of andrographolide and neoandrographolide in leaf and callus samples were based on comparison of retention times in sample and reference (standard) chromatograms. HPLC analysis was done using an Ultimate 3000 HPLC coupled to a CAD detector. Separation was carried out using a Phenomenox Hyperclone (150 X 4.6 mm; 5μ) C-18 column with water and acetonitrile as the mobile phase. The following gradient was used for the chromatographic analysis: 20%-50% acetonitrile from 0-50 min. An injection volume of 10 μL and a column temperature of 25°C

was used. The concentrated leaf extracts were diluted 4-fold before injection. The callus extracts were injected in their concentrated form.

In order to quantify the amount of andrographolide and neoandrographolide in the leaves and callus, a calibration curve was made from stock solutions of the two standards. The sample concentrations for the calibration curve were 1000 **µg**/mL methanol, 500 **µg**/mL, 250 **µg**/mL, 125 **µg**/mL, 62.5 **µg**/mL, 31.25 **µg**/mL, and 15.62 **µg**/mL. The peak areas of these samples were graphed and linearized. Comparing the HPLC results for the peak areas of leaf and callus samples to this curve gave the percentage (by weight) of the andrographolide and neoandrographolide content in the samples.

Results

Standards

The HPLC analysis of the standards showed that the retention time was approximately 10.5 minutes for andrographolide and 19.1 minutes for neoandrographolide as shown in Figures 2 and 3. When a mixture of the two compounds was analyzed, each presented a separate, distinct peak, as shown in Figure 4.

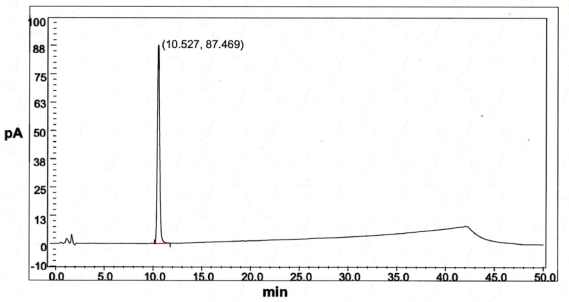

Figure 2: HPLC Results for Andrographolide Standard (1 mg/mL).

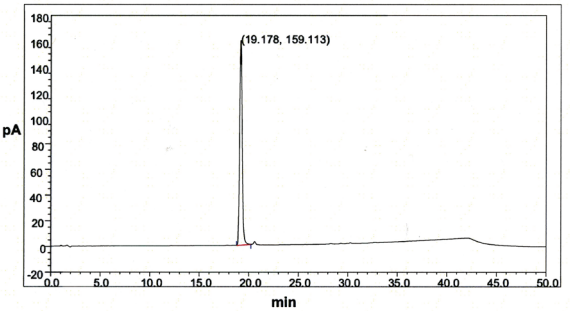

Figure 3: HPLC Results for Neoandrographolide Standard (1 mg/mL).

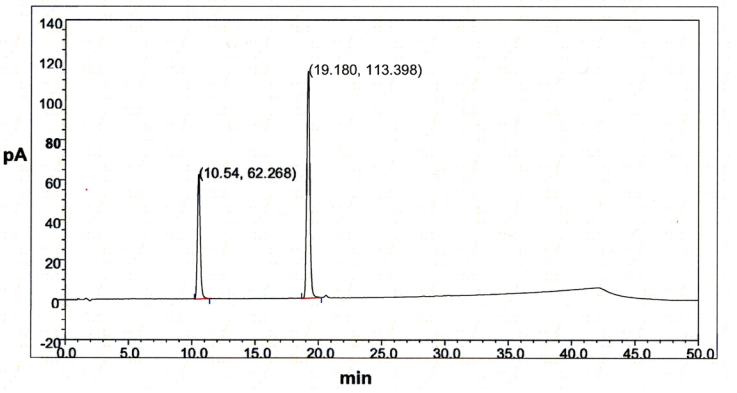

Figure 4: HPLC Results for 1:1 Ratio of Andrographolide to Neoandrographolide (1

Leaves and Calluses

All three samples of leaf tissue contained amounts of neoandrographolide and andrographolide. The two peaks labeled in Figures 5, 6, and 7 are the peaks representing andrographolide and neoandrographolide content. The calibration curves for each neoandrographolide and andrographolide are represented by Figures 8 and 9, respectively. Using the information from Figures 8 and 9, the percent weight of each secondary metabolite was calculated and results are presented in Table 1. The callus samples are represented by Figures 10, 11, and 12. None of the callus samples contained traces of either andrographolide or neoandrographolide.

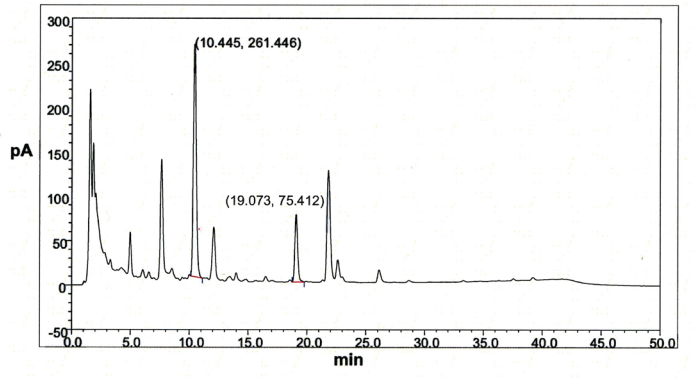

Figure 5: HPLC Results for Leaf Tissue Sample 1 (1 g dried leaf tissue/5 mL methanol).

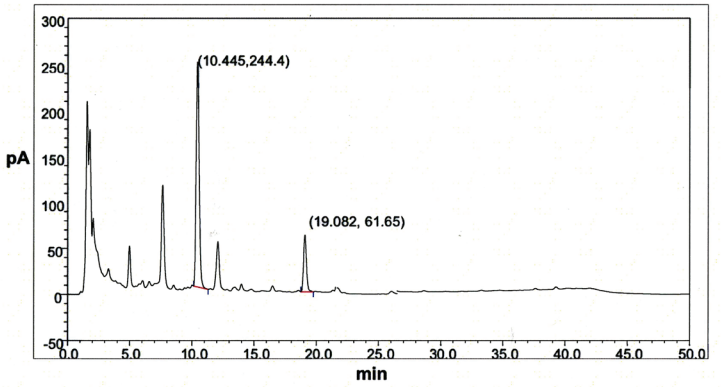

Figure 6: HPLC Results for Leaf Tissue Sample 2 (1 g dried leaf tissue/5 mL methanol).

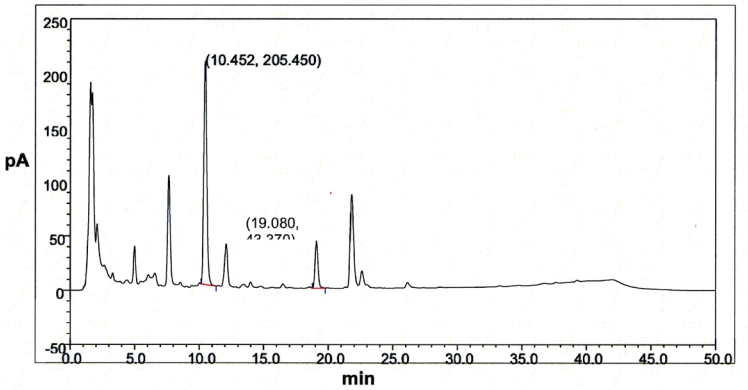

Figure 7: HPLC Results for Leaf Tissue Sample 3 (1 g dried leaf tissue/5 mL methanol).

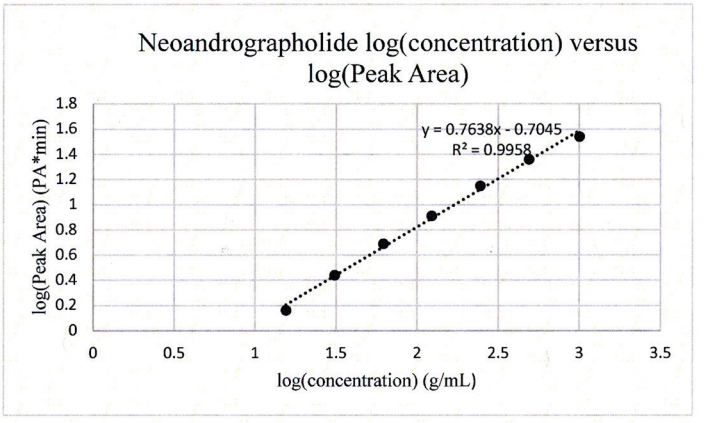

Figure 8: Calibration Curve for Neoandrographolide.

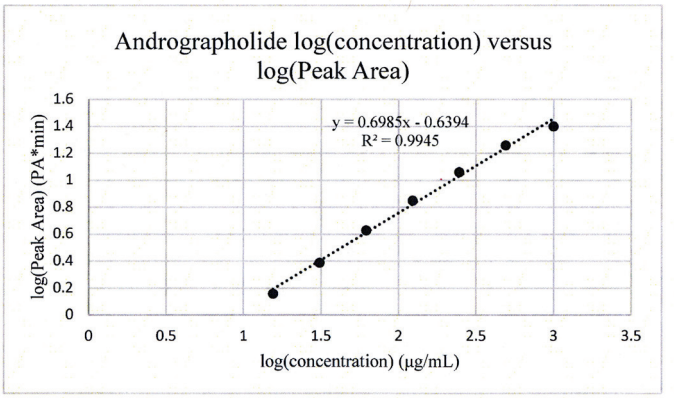

Figure 9: Calibration Curve for Andrographolide.

Table 1: Percent Weight of Andrographolide and Neoandrographolide in Leaf Samples.

	Andrographolide % w/w	Neoandrographolide % w/w
Leaf 1	0.05	0.002
Leaf 2	0.42	0.002
Leaf 3	0.29	0.001
Mean± SE	0.253± 0.108	0.0017± 0.0003

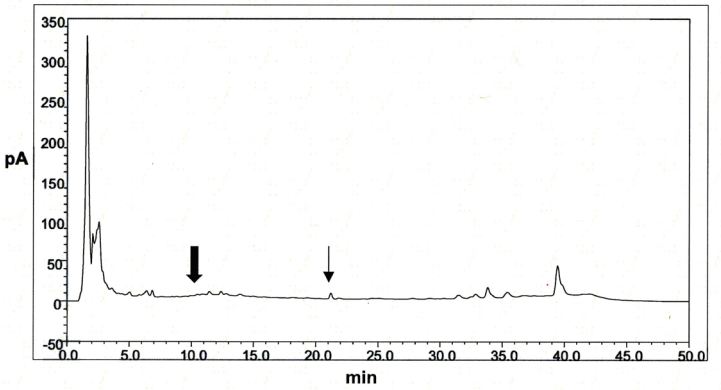

Figure 10: HPLC Results for Callus Tissue Sample 1 (1 g dried callus/5 mL methanol).

(⬇= Andrographolide Peak Location; ↓ Neoandrographolide Peak Location)

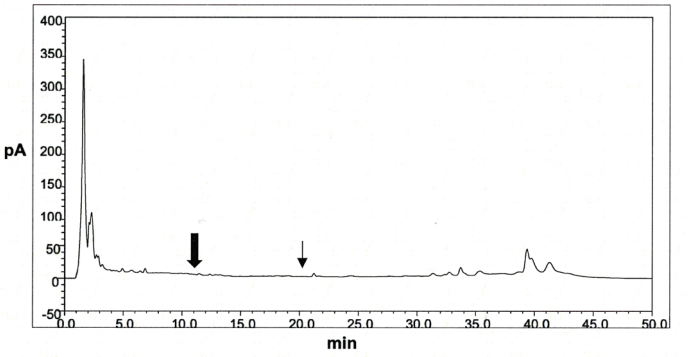

Figure 11: HPLC Results for Callus Tissue Sample 2 (1 g dried callus/5 mL methanol).

(↓ = Andrographolide Peak Location; ↓ Neoandrographolide Peak Location)

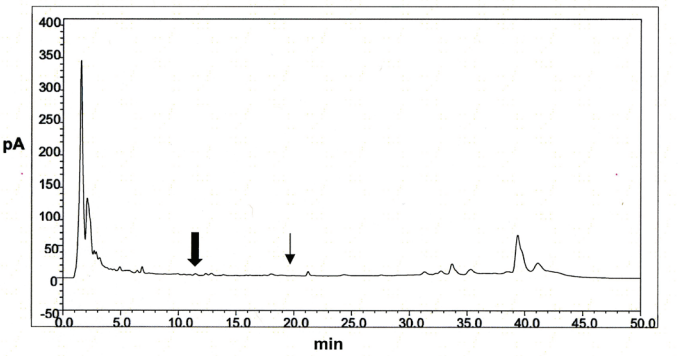

Figure 12: HPLC Results for Callus Tissue Sample 3 (1 g dried callus/5 mL methanol).

(↓ = Andrographolide Peak Location; ↓ Neoandrographolide Peak Location)

Discussion

Like the Chatterjee experiment where the leaf tissue was removed during the winter months, the % w/w results are similar for andrographolide and neoandrographolide in leaf tissue taken from a transferred plant in greenhouse conditions.[5] The lack of andrographolide in callus tissues was expected based on the Ananthi experiment[3] and our current knowledge of the workings of callus.

The results of this experiment are in line with what is known about the ability of callus to produce secondary compounds. In many cases, callus tissue cultures have successfully produced various secondary metabolites. *In vitro* studies of ginseng are a well-known example of the success of calli producing secondary compounds, such as saponin.[8] However, many callus tissue cultures do not produce

secondary compounds.[8] For example, *Hypericum perforatum* callus tissues have not successfully produced the secondary compounds hypericins and hyperforins, which are produced in the plant.[8]

Callus may not produce secondary compounds because of a lack of elicitors.[3] Secondary metabolites are naturally produced by plants as a defense response against pathogens. Once attacked, elicitors in the plant are activated and act as a signal for the plant to produce secondary metabolites.[3] *Ammi majus L.* callus is an example of a tissue culture that does not produce traceable amounts of a secondary compound, umbelliferone, because it lacks the elicitor needed, benzo(1,2,3)-thiadiazole-7carbothionic acid S-methyl ester. Once this elicitor was introduced, the callus began producing umbelliferone.[3]

There is currently no information on *Andrographis paniculata* callus tissue's production of neoandrographolide. There are, however, many studies on the health benefits and usage of this secondary metabolite created by the plant's leaves.[4] With knowledge of how this plant's callus tissue reacts in a standard medium without elicitors, the next step would be to grow the callus in media impregnated with various elicitors and record how this affects production of neoandrographolide. Frequently used elicitors include fungal carbohydrates, yeast extract, methyl jasmonate and chitosan.[8] Other options to induce secondary metabolites include, genetic manipulation, metabolic engineering, and the use of symbionts.[1]

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