The Effects of *Hexastylis arifolia* Extract on PC12 Cell Cultures

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ABSTRACT

*Hexastylis arifolia* has been traditionally used by the Lumbee Native Americans as a flavoring in tonics and for the treatment of heart problems. Because of the medicinal folklore behind this plant, we have chosen to scientifically analyze any potential neuroprotective properties of the plant. Upon running the root extract of the plant through a gas chromatography mass spectrometer, some compounds, such as asarone, methyleugenol, and 5,2,4,5-trimethoxybenzaldehyde, were identified. The results of viability assays of the oil extract on PC12 cells suggest that the extract has negative affects of cell viability in a dose- and time-dependent manner. For the smaller concentrations, we suggest that western blot assays test for the increased expression of brain derived neurotrophic factor (BDNF) for future tests.

BACKGROUND

The genus *Hexastylis* is a low, evergreen, aromatic group of plants related to wild ginger (*Asarium*) (Blomquist, 1957). This genus has nine species, which are endemic only to the Southeastern United States (Soltis, 1984). An Internet search will yield websites either recommending this genus as a substitute for ginger in recipes or warning against its use due to toxic claims. The species of interest in this paper is *Hexastylis arifolia* (*H. arifolia*), also known as the “heartleaf” or “little brown jugs” due to the shape of its leaves and flowers. *H. arifolia* has been traditionally used as a flavoring in tonics and for the treatment of heart problems among the Lumbee Native Americans (Boughman and Oxendine, 2004).

Many plant species worldwide are increasingly threatened due to deforestation trends. Before the loss of species with medicinal or economic potential, it is important to scientifically examine their value (Bruke, 2001). Because of the medicinal folklore of the Lumbee associated with *H. arifolia*, this
The study aims to evaluate potential benefits from the essential oils extracted from this plant. An analysis of the chemical composition of *H. arifolia* describes the presence of many bioactive compounds. Oil extraction of the roots have revealed the top four chemicals found in *H. arifolia* by percent are safrole (58.2%), methyleugenol (19.9%), (trans)-methylisoeugenol (8.7%), and ß-asarone (3.3%) (Hayashi et al., 1983). Experiments have shown that safrole is a carcinogen in live rats (Yu et al., 2010). Alternatively it can also induce dose- and time-dependent apoptosis in human leukemia cells, cancerous human tongue cells, and cancerous human oral cells *in vitro* (Yu et al. 2012; Yu et al., 2011; Yu et al. 2010). These studies also suggest that the apoptosis is a result of caspase-dependent signaling pathways. Experiments performed by Yu et al. in 2011 indicated that safrole decreased Bcl-2 in cell cultures. They propose that the decrease of Bcl-2 causes cytochrome c release from the mitochondria after mitochondrial membrane potential collapse. Cytochrome c in turn activates caspase-9, which leads to the activation of caspase-3, resulting in apoptosis (Yu et al., 2011). This induced apoptosis makes safrole a good candidate for anti-cancer research (Yu et al., 2010). If anti-cancerous properties are confirmed, *H. arifolia* can potentially be a welcome source of cultivating safrole.

Interestingly, the effects of safrole could be confounded by the presence of ß-asarone in the extract. According to a study done by Li et al. in 2010, cells treated with ß-asarone reversed the down regulation of the Bcl-2 protein family induced by ß-amyloid. The Bcl-2 protein family is anti-apoptotic due to its involvement in regulation of the mitochondrial membrane potential (Yu et al., 2011). Additionally, studies done by Dong et al. in 2014 indicated that ß-asarone increases brain-derived neurotrophic factor (BDNF) expression in the hippocampus of mildly stressed rats. BDNF is an important protein involved in the development, maintenance, and protection of neural cells (Kondo et al. 2013). Therefore, due to the presence of ß-asarone, *H. arifolia* extract has the potential to have neuroprotective qualities.

In addition, safrole, ß-asarone, and methyleugenol also have the potential to display mutagenic or carcinogenic properties (Yu et al., 2010; Unger and Melzig, 2012; Herrmann, et al., 2013).

The overall purpose of this project is to study the effects a methanol extract from *H. arifolia* *in vitro* in order to gain insight on the effect *in vivo*. Rat pheochromocytoma (PC12) cells have the ability to differentiate into neuron-like cells and for that reason were chosen for this project (Wang et al., 2006). Upon researching each bioactive compound, we hypothesized that PC12 cells treated with *Hexastylis arifolia* root essential oil extract would undergo induced apoptosis in a time- and dose-dependent manner. In this study, essential oils were extracted from samples of *H. arifolia* and analyzed for their chemical composition. PC12 cells were then treated with various concentrations of the extract and tested for viability in 24, 48, and 72-hour increments.

**MATERIALS AND METHODS**

**Cell Culture**

PC12 cells were supplied as a generous gift from Karina Ricart at UAB. 100mm plates were collagen-coated, 1:100 in 30% ethanol under aseptic conditions. Cells were grown in RPMI media containing 10% fetal bovine serum, 5% horse serum, and 1% penicillin/streptomycin and incubated at 37ºC under
humidified conditions with 5% CO₂. Cells were split when they reached 70% confluence (every three to four days).

**H. arifolia Oil Extraction**

Two samples were collected from the nearby woods of the Samford University campus. The plants were rinsed with tap water then desiccated in a Lane Science Equipment Co. dryer for five days at approximately 45ºC. The roots of the samples were ground with a mortar and pestle into a fine powder. The first sample yielded 7.1g and the second yielded 3.7g. The powder was left to soak in a 10:1 methanol to powder weight ratio for 24 hours. The excess grounds were removed through use of a 7µm filter paper. The remaining compounds in the methanol solution were extracted using solid phase extraction (SPE) procedures and collected in glass vials. All water used from this point was distilled. The methanol solution was diluted 3:10 methanol with water. New SPE tubes were used for each sample and were conditioned using 10mL prewash of 30% ethanol and 10mL rinse of 20% ethanol solutions. Sample one was run through the SPE tubes and collected by running 1mL of three different elutions. Elution one was 66% water and 33% methanol. Elution two was 33% water and 66% methanol. Elution three was 100% methanol. Sample two was run and collected by running 2mL of methanol. All vials of all samples were left to dry, leaving the solid extract. Once dry, the vials were capped and the extract was stored at room temperature.

**Extract Composition Analysis**

Elution three (100% methanol elution) was resuspended in ethyl acetate (3.6mg/mL) and run though a Thermo Scientific Trace 1310 gas chromatography and ISQ mass spectrometer (GC/MS). The GC/MS was run with 1µL injection with Helium gas as the carrier. The flow rate was 60mL/min. The column temperature gradient was 100ºC for five minutes, solvent delay for two minutes, ramp up to 300ºC at 10ºC/min, and maintain 300ºC for five minutes.

**Extract Viability**

The extract was resuspended in ethanol and stored at 2ºC. Three 96 well plates were collagen-coated 1:100 in 30% ethanol under aseptic conditions. PC12 cells were plated 1x10³ per well. All solutions contained 0.25M concentration of ethanol. Concentrations of 276µg/ml, 138µg/mL, 69µg/mL, 34µg/mL, 17µg/mL and 0µg/mL were added to the well plate in replicates of 8 wells each. An MTT-assay was performed 24, 48, and 72 hours following treatment using a Beckman Coulter AD340 plate reader with filter set to 570nm. The test was repeated for a total of three trials.

**RESULTS**

**Extract Composition Analysis**
GC/MS ran for 30.10 minutes and was able to detect the presence of multiple compounds shown as peaks in Figure 1. The mass spectrometric data collected at 12.40 minutes yielded peaks at 208, 193, 165, and 69 m/z, which are indicative of the ion fragments of asarone as seen in Figure 2 (Bruke, 2001). The data collected at 10.96 minutes yielded peaks at 208, 193, 177, and 77, which indicate the ion fragments of elemicin (National Institute of Standards and Technology). The data collected at 8.20 minutes yielded peaks at 178, 163, 147, 103, and 91 m/z, which are indicative of the ion fragments of methyleugenol (Spectral Database for Organic Compounds). The data collected at 9.79 minutes yielded peaks at 178, 163, 147, and 107 m/z, which are indicative of methylisoeugenol (National Institute of Standards and Technology). The data collected at 12.99 minutes yielded peaks at 196, 181, 150, 125, and 69 m/z, which are indicative of 5,2,4,5-trimethoxybenzaldehyde (Bruke, 2001). Of the remaining peaks, no evidence of the presence of safrole was obtained (Table 1).

**Extract Viability**

The average absorption of the control was calculated for each trial. The percent viability was calculated by multiplying the absorption by one-hundred and dividing by the average absorption for each trial ((absorption x 100)/average absorption of control for trial). Two way ANOVA was run using R version 3.1.1 for Mac OS X. The overall effect of higher concentration was decreased viability F(5,408)= p<0.001. All concentrations were compared to the control. For the 24 hour trials, there was no statistical difference for 17µg/mL (p=0.9995), 34µg/mL (p=0.9956), 69µg/mL (p=0.9966), 138µg/mL (p=0.9689). There was a statistical difference for 276µg/mL (p=0.0023) (Figure 1). For the 48 hour trials, there was no statistical difference for 17µg/mL (p=0.9999) and 34µg/mL (p=0.8631). There was a statistical difference for 69µg/mL (p=0.0070), 138µg/mL (p=0.0068), and 276µg/mL (p=0.0000) (Figure 2). For the 72 hour trials, there was no statistical difference for 17µg/mL (p=0.9999) and 34µg/mL (p=0.5855). There was a statistical difference for 69µg/mL (p=0.0000), 138µg/mL (p=0.0000), and 276µg/mL (p=0.0000) (Figure 2) (Figure 3).

**DISCUSSION**

While indigenous to the entire Southeastern United States, efforts to obtain wild ginger from Oak Mountain State park were unsuccessful. Therefore samples were taken from the nearby woods of Samford University. Analysis of the extract composition suggests the presence of asarone, elemicin, methyleugenol, isomethyleugenol, and 5,2,4,5-trimethoxybenzaldehyde. These compounds are all very similar in structure and have been previously reported in *H. arifolia* oil extractions. Evidence for the compounds listed in the extract composition analysis should be further validated by isolating the compounds from the extract and then testing them by use of H-NMR, C-NMR, and UV spectroscopy. This is due to the fact that pairs of compounds are structurally similar and share similar peaks. Among these pairs is asarone and elemicin.

The essential oil extraction analyzed by Hayashi et al. reported the presence of safrole (58.2%), methyleugenol (19.9%), (trans)-methylisoeugenol (8.7%), ß-asarone (3.3%), and elemicin (0.4%). The relative size of the peaks from our GC/MS data suggest that asarone and elemicine were among the
highest concentrations in our extraction. There was a lack of evidence for the presence of safrole. This could be due to the method of extraction done in this experiment. Hayashi et al. used ethyl acetate to perform their extraction whereas we used methanol. According to another study done by Bruke in 2001, the relative quantities of compounds found in a methanol extraction were β-asarone (42.7%), methyleugenol (34.6%), methylisoeugenol (15.6%), and safrole (3.5%). It is possible that safrole was present but that the solution we ran through the GC/MS was too diluted, and the solvent covered the safrole peak.

Our MTT viability assay results indicate that the extract decreases cell viability in a dose- and time-dependent manner. The slope for percent cell viability versus concentration decreased as time increased (as can be compared between Figure 1, Figure 2, and Figure 3). It was also noted that as time increased p-values dropped for concentrations. Possible explanations include the fact that both isomers of asarone, β- and α-, show cytotoxic effects in cell culture (Unger and Melzig, 2012). As stated previously, further tests would be needed in order to validate the presence of asarone but the relative quantities of α- and β-asarone would give a better insight on the results of the viability. However, the similar mass spectra of the isomers make identification difficult and would require reference compounds (Zuo et al., 2011). According to Bruke the percent presence of β-asarone should be about 42.7% from a methanol extraction. If this is the case, the majority of the asarone detected would be β-asarone. A study done by Liu et al. in 2013 reported that although β-asarone has been reported as a potential carcinogen, the LD50 is 1.56g/kg and should be safe for clinical use in small quantities. In other studies, β-asarone has been linked to BDNF expression in the hippocampus of mildly stressed rats (Dong et al., 2014). BDNF is involved in neuroplasticity and aids in maturation, protection, and maintenance of neurons. This quality could give it the potential to be used as an anti-depressant (Dong et al., 2014). Other studies concerning the increased regulation of BDNF by β-asarone suggest the potential of this molecule to slightly reverse the effects of Alzheimer’s Disease (Yang et al., 2013; Li et al., 2012; Li et al., 2010). In order to test for increased expression of BDNF in PC12 cells a western blot would have to be performed using anti-BDNF antibodies. A positive correlation between extract treatment and BDNF expression could suggest neuroprotective qualities of H. arifolia.

WORKS CITED


Table 1. A list of the identified materials from the gas chromatography mass spectrometry run from the extract suspended in ethyl acetate (3.6mg/mL). Retention time is listed as well as molecular ion peak. Some compounds were not identified.

<table>
<thead>
<tr>
<th>Literature Match</th>
<th>Retention Time (minutes)</th>
<th>Molecular Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asarone</td>
<td>12.40</td>
<td>208</td>
</tr>
<tr>
<td>Elemicin</td>
<td>10.96</td>
<td>208</td>
</tr>
<tr>
<td>Methyleugenol</td>
<td>8.20</td>
<td>178</td>
</tr>
<tr>
<td>Methylisoëugenol</td>
<td>9.79</td>
<td>178</td>
</tr>
<tr>
<td>5,2,4,5-trimethoxybenzaldehyde</td>
<td>12.99</td>
<td>196</td>
</tr>
<tr>
<td>Unknown</td>
<td>13.08</td>
<td>104</td>
</tr>
<tr>
<td>Unknown</td>
<td>20.87</td>
<td>207</td>
</tr>
<tr>
<td>Unknown</td>
<td>14.18</td>
<td>188</td>
</tr>
<tr>
<td>Unknown</td>
<td>19.79</td>
<td>207</td>
</tr>
</tbody>
</table>
Figure 1. 24 hour extract viability. Asterisk denotes a significant difference between that concentration and the control (p-value less than 0.01). Double asterisks denote a significant difference between that concentration and the control (p-value less than 0.0001). For the 24 hour trials, there was no statistical difference for 17µg/mL (p=0.9995), 34µg/mL (p=0.9956), 69µg/mL (p=0.9966), 138µg/mL (p=0.9689). There was a statistical difference for 276µg/mL (p=0.0023)
Figure 2. 48 hour extract viability. Asterisk denotes a significant difference between that concentration and the control (p-value less than 0.01). Double asterisks denote a significant difference between that concentration and the control (p-value less than 0.0001). For the 48 hour trials, there was no statistical difference for 17µg/mL (p=0.9999) and 34µg/mL (p=0.8631). There was a statistical difference for 69µg/mL (p=0.0070), 138µg/mL (p=0.0068), and 276µg/mL (p=0.0000)
Figure 3. 72 hour extract viability. Asterisk denotes a significant difference between that concentration and the control (p-value less than 0.01). Double asterisks denote a significant difference between that concentration and the control (p-value less than 0.0001). For the 72 hour trials, there was no statistical difference for 17µg/mL (p=0.9999) and 34µg/mL (p=0.5855). There was a statistical difference for 69µg/mL (p=0.0000), 138µg/mL (p=0.0000), and 276µg/mL (p=0.0000) (Figure 2)