Does Monotropa uniflora produce grayanotoxin?

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Abstract

Finding new pain management therapies will mitigate the effect addictive opioids have on our society. One folk medicine that shows promise as a pain management tool is Monotropa uniflora. M. uniflora is a member of the Ericaceae family of plants, many of which produce a neurotoxin called grayanotoxin (GTX). The primary purpose of the experiment is to determine if the alcohol extraction of M. uniflora affects MCF-7 cells in the same way as other Ericaceae family plants that produce GTX to explain the reaction people reportedly have with M. uniflora extract. Previous studies concerning M. uniflora were studying antimicrobial activity of the ethanol extract, among other solvents. The physiological effects of M. uniflora extract on human cell culture have largely been unexplored in western literature. By studying the dosage effect of the ethanol extract in human breast cancer (MCF-7) cells, the activity of M. uniflora extract can begin to be characterized via Raman spectroscopy. Preliminary findings suggest that the M. uniflora ethanol extract effects the MCF-7 cells independently of the ethanol solvent. Additional analysis is continuing to expand effects across time to determine if the cells are metabolizing the extract and solvent differently. Comparing Raman characterization between M. uniflora extract with characterization of GTX in MCF-7 cells will give further evidence to determine whether M. uniflora produces GTX and lend to future research determining if either are safe alternatives to opioid analgesics.

Introduction

Monotropa uniflora, commonly called “ghost pipe”, is a white holoparasitic plant that thrives in lower pH soils where wild low-bush blueberries can be found. Such habitats where the presence of *M. uniflora* have been recorded are older, secondary growth deciduous forests with full canopy cover and a robust mycelium community in a mutualist relationship with the trees (1). Current research studies focus on *M. uniflora* in plant diversity surveys (2,3) or are concerned with trying to grow the plant in laboratory settings (4,5). An older paper from 1951 studies the use of various solvent extractions of many plants for antimicrobial activity. Extracts of *M. uniflora* were placed in the top ten most antimicrobial plants (6). Only one source from 1889 determined that *M. uniflora* may contain a neurotoxin (7) but has not been reproduced because the methods of extraction have changed and improved significantly since 1889. There has not been enough investigation into potential therapeutic properties of *M. uniflora* despite many non-biomedical sources suggesting that the plant has pain relieving properties (8–10). A common consensus between homeopathic practitioners, folk medicine authors, and YouTube videos is that ghost pipe

is used to treat pain, anxiety, and insomnia (8–13), however, there is little to no literature further confirming the presence of grayanotoxin in *M. uniflora*.

The plant is used as an antinociceptive, anti-anxiety and anti-insomnia medicine, these effects could be explained by GTX. The mechanism for GTX has been demonstrated as binding to sodium voltage channels in cells, causing depolarization at a resting state in nerves (14). This necessitates stronger stimuli to meet the activation threshold, resulting in lowered nociceptive and sympathetic system communication. Four of the common grayanane diterpenoids studied have varying toxicities and efficacies. Sun et al. report that GTX-1 and GTX-4 showed analgesic properties more potent than morphine (15).

Figure 1.

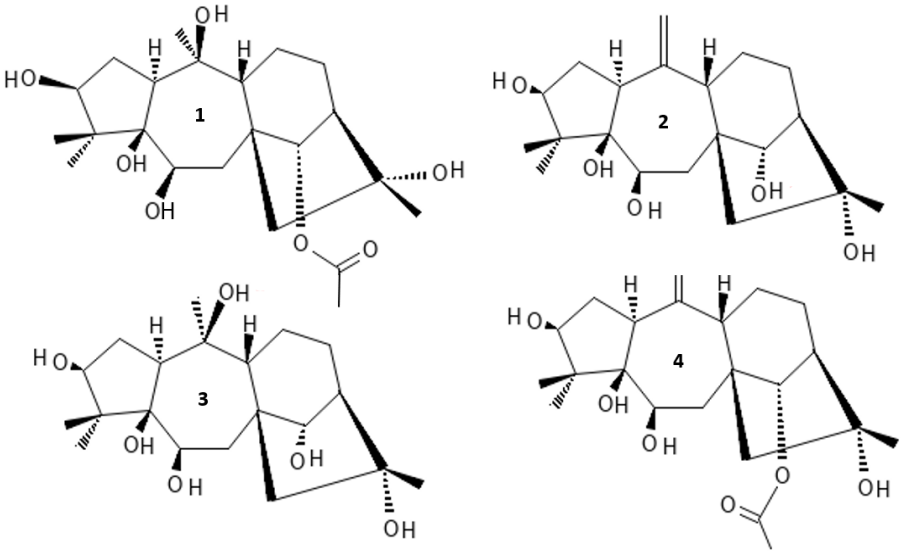

Figure 1. Structures of grayanotoxin 1 through 4. Figure 1 was made using the National Institutes of Health PubChem Sketcher V2.4.

The major difference between GTX-1 and GTX-3 is the hydration of the acetate group under the ring, allowing the acetate to leave due to warmer temperatures (16), shown by Figure 2.

Figure 2.

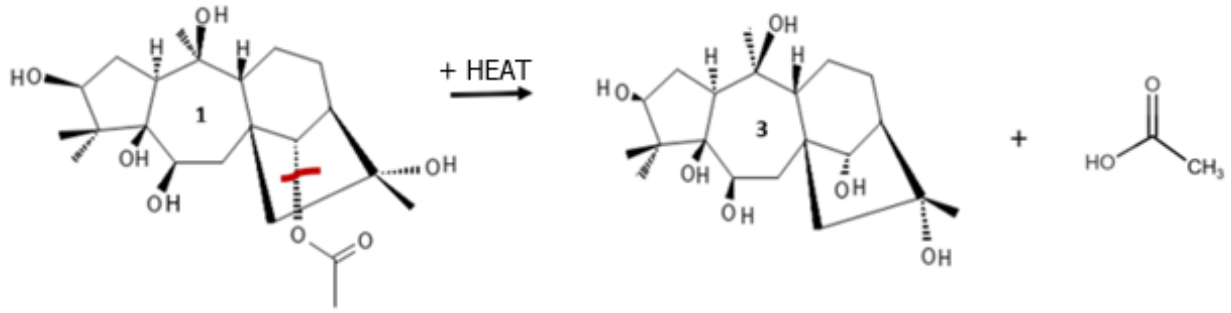

Figure 2. Heat causes the ester group to leave the ring, turning GTX-1 into GTX-3. Figure 2 was made using the National Institutes of Health PubChem Sketcher V2.4.

GTX naturally occurs in other Ericaceae plants such as *Kalmia latifolia* (mountain laurel) and Rhododendrons (15,17–20). Rhododendron sourced GTX is currently being studied in the form of Rhododendron honey in the Middle East. From over 1,199 case studies, the average dose people take of Rhododendron honey (RH) is 20 to 100 grams, used mainly to self-treat hypertension and sexual enhancement. There are no records of people dying from an overdose of RH since the 1800s, as those who are admitted to the emergency room present with a low pulse rate and a drop in blood pressure; these symptoms are treated with atropine after confirming RH consumption (21). Besides RH, GTX can be obtained by exhaustive extraction from Rhododendron leaves. in 1889, Professor Plugge noted that andromedotoxin (GTX) is highly soluble in ethanol (7), as seen in contemporary studies which use an exhaustive ethanol extraction process described by Lechtenberg et al. (18), Li et al. (19), and Sun et al. (15). While Rhododendrons produce abundant plant material to extract from, *M. uniflora* does not. Since the effect of GTX on cells is known, a comparison can be made between cells in 2D culture dosed with an ethanol extract of *M. uniflora* and cells dosed with GTX.

Methods

Raman spectroscopy can detect subtle biochemical changes in the composition of biological samples, which can overcome the limitation imposed by the relative rarity of *M. uniflora*. Visible light lasers are used to excite the covalent bonds between atoms, which allows Raman spectroscopy to detect biochemical markers without incurring damage to the sample. While Raman spectroscopy cannot detect changes in ion content directly, it can detect changes in the bond stretch of water molecules as a result of ionic solute changes, indicating cell depolarization due to GTX introduction into the cell media.

MCF-7 human breast cancer cells are well characterized by Raman spectroscopy (22–26), which are hardy in 2D culture environments and have a quick doubling rate, making MCF-7 cells suitable for dose studies focusing on small cellular changes under Raman spectroscopy.

Ethanol extracts are commonly called tinctures in alternative medicine and can be made by the general population (11-13). Since tincturing is the common method of preparation and consumption for *M. uniflora,* a tincture is used in this study similar to RH studies (27–30).

Limitations in RH studies showed that RH varied in concentration of GTX, which produced a large variance of signal. However, since the subjects of RH studies were living animals, more GTX needed to be present to give consistent results. There may be more GTX present in the aerial part of *M. uniflora* on average compared to relative amounts of Rhododendron nectar in RH collected by bees in the Black Sea region.

M. uniflora tincture was prepared by sustainably harvesting the aerial parts of fresh flowers from July to October, sectioning and placing into a container to fill ¾ by volume, moderately packed. Ninety percent ethanol was poured into the container approximately one inch above the plant material and agitated twice weekly in storage for 6 months in a dark, cool, dry space for extraction. The transparent and clear ethanol turned black and cloudy during extraction time.

Experimental design 1: to determine if M. uniflora extract has any activity on MCF-7 cells.

MCF-7 cells were seeded in T-25 flasks in high glucose Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal bovine serum, incubated at 37°C in an atmosphere of 5% CO_2. The ethanol concentration of the ethanol solvent group and the *M. uniflora* tincture concentration were kept the same at 0.225% ethanol by volume per internal lab protocols. After proliferation to 80-100% confluency, 3 replicates of 3 treatment groups were tested: null treatment, ethanol treatment (OH), and extract treatment (EXT). Three replicates were exposed to the treatments for 10 minutes and 2 days after a 24-hour period when the treated media was refreshed. Each sample was fixed to aluminum Raman slides with 70% ethanol and scanned using a 532 nm wavelength laser for 8 replicates of 20-second exposure.

Experimental design 2: to determine M. uniflora time-course effects.

MCF-7 cells were seeded and split into null-control group, OH group and EXT group similar to the first experiment. However, the times of exposure were changed to 20-minute intervals until 80 minutes had elapsed. Raman spectra of each sample were taken in 15 replicates under the same conditions as the first experiment.

Results

The Raman spectra were analyzed with the unsupervised multivariate analysis (MVA) technique, principal component analysis (PCA), and the supervised MVA technique linear discriminant analysis (LDA) program to return a scatterplot, known as a scores plot, to determine if there are differences between groups. Points that appear close together on a scores plot have similar spectral similarity, and thus biochemical similarity. This approach allows for the visualization of clustering of cellular biochemistry on a 2D plot as seen in figure 3.

Figure 3.

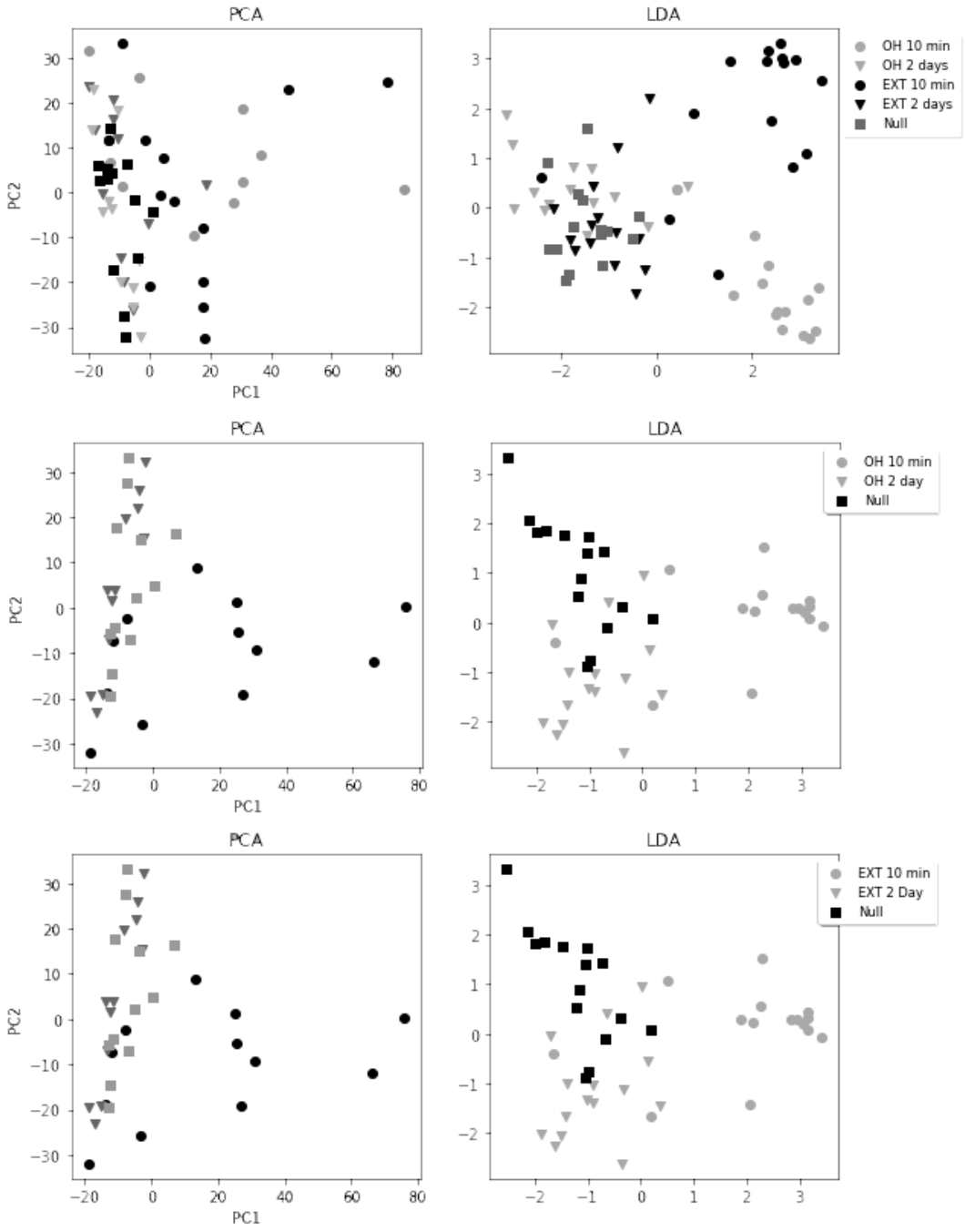

Figure 3 shows the PCA/LDA scatter plot results from experiment 1. The 10-minute extract treatment group (EXT) showed significant differences from both the null group and the 2-day EXT exposure group. The 10-minute ethanol solvent group (OH) also shows significant differences from both the null group and the 2-day OH exposure group. When all groups are examined together, both the 2-day EXT and OH groups are similar to the null group. However, the EXT and OH 10-minute groups are significantly different from each other as well as the null and 2-day exposure groups. These results show that the *M. uniflora* extract has some effect on the MCF-7 cells that are different than the ethanol solvent.

Figure 4.

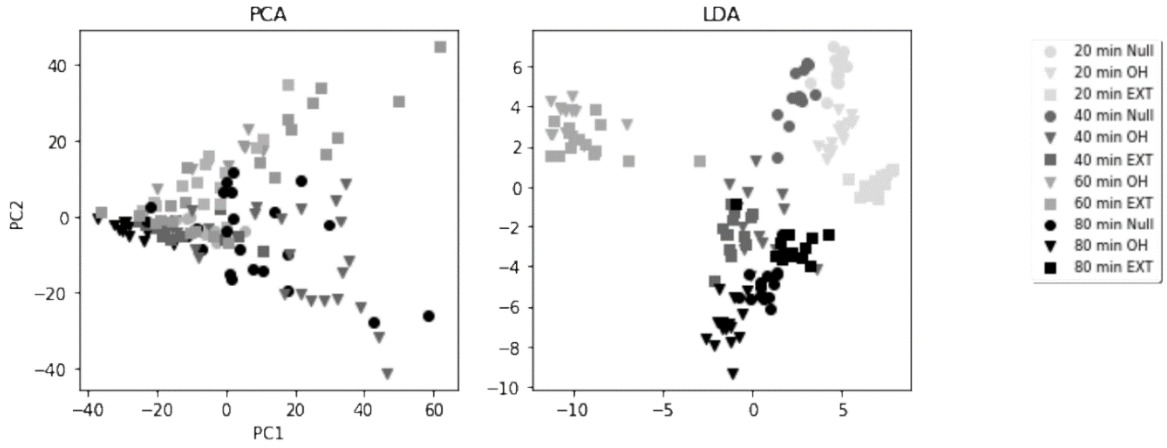

Figure 4 shows the PCA/LDA results of experiment 2. The 20-minute exposure OH and EXT groups cluster apart from each other and other groups in the LDA plane, indicating unique differences within the 20-minute exposure groups and differences from all other groups. The 20-minute time trial corroborates the results of the first experiment. The 20- and 40-minute null groups are clustered near each other in the LDA plane, showing similarity with each other, which is expected. The 40-minute exposure OH and EXT groups show some overlap with each other, and cluster near the 80-minute exposure groups. The 60-minute null group cells were observed to be yellow in color in a pile on the slide that was denser than the other samples, unable to be usefully scanned without fluorescence. The 60-minute exposure OH and EXT groups were also observed to be yellow, while the other time groups were colored white on the slides and were able to be scanned. Both 60-minute groups seem to be an outlier, clustering on the far left side of the LDA plane. The 80-minute null group appears lower in the LDA plane, with the EXT and OH groups clustered tightly together, but on opposite sides of the 80-minute null group, indicating a clear difference between the three 80-minute groups.

Figure 5.

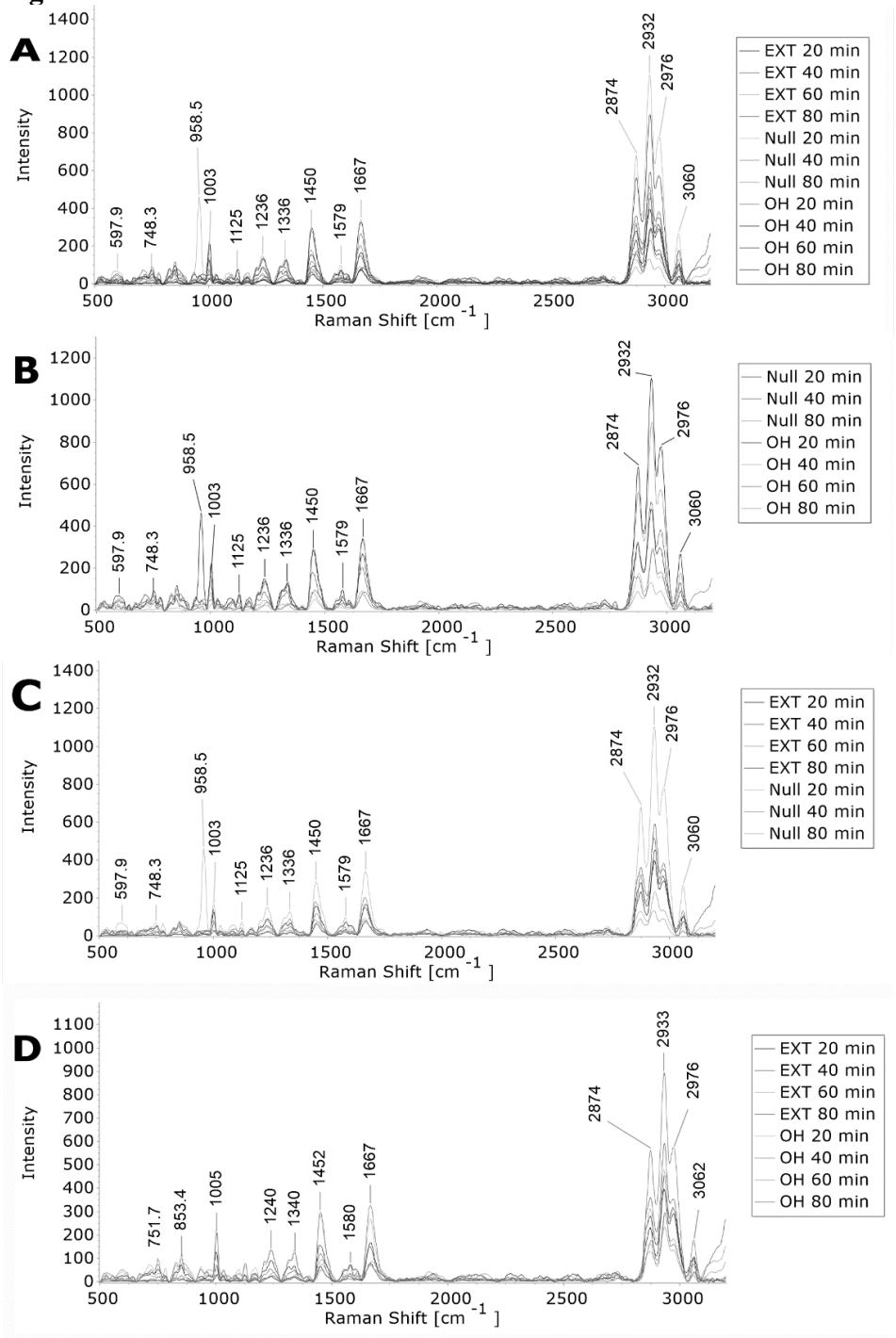

Figure 5 is the collection of MCF-7 cell Raman spectra gathered using a 532 nm laser, 50x objective, and the smallest hole setting for 8 captures using 20 second exposure for 15 scans of each sample. Sample spectra were baselined, smoothed, and merged in Spectragryph software. Each plot shows the average group spectra plots with axis Raman shifted wavelength (cm^{-1}) by intensity of signal for the null, ethanol solvent (OH) and *M. uniflora* extract (EXT) groups at each time exposure (panel A), null and OH groups at each time in panel B, EXT and null in panel C, and EXT and OH groups in panel D. All spectra share nearly the same significant peaks, with

the 20-minute null group introducing a single peak at 958.5 cm^{-1}.

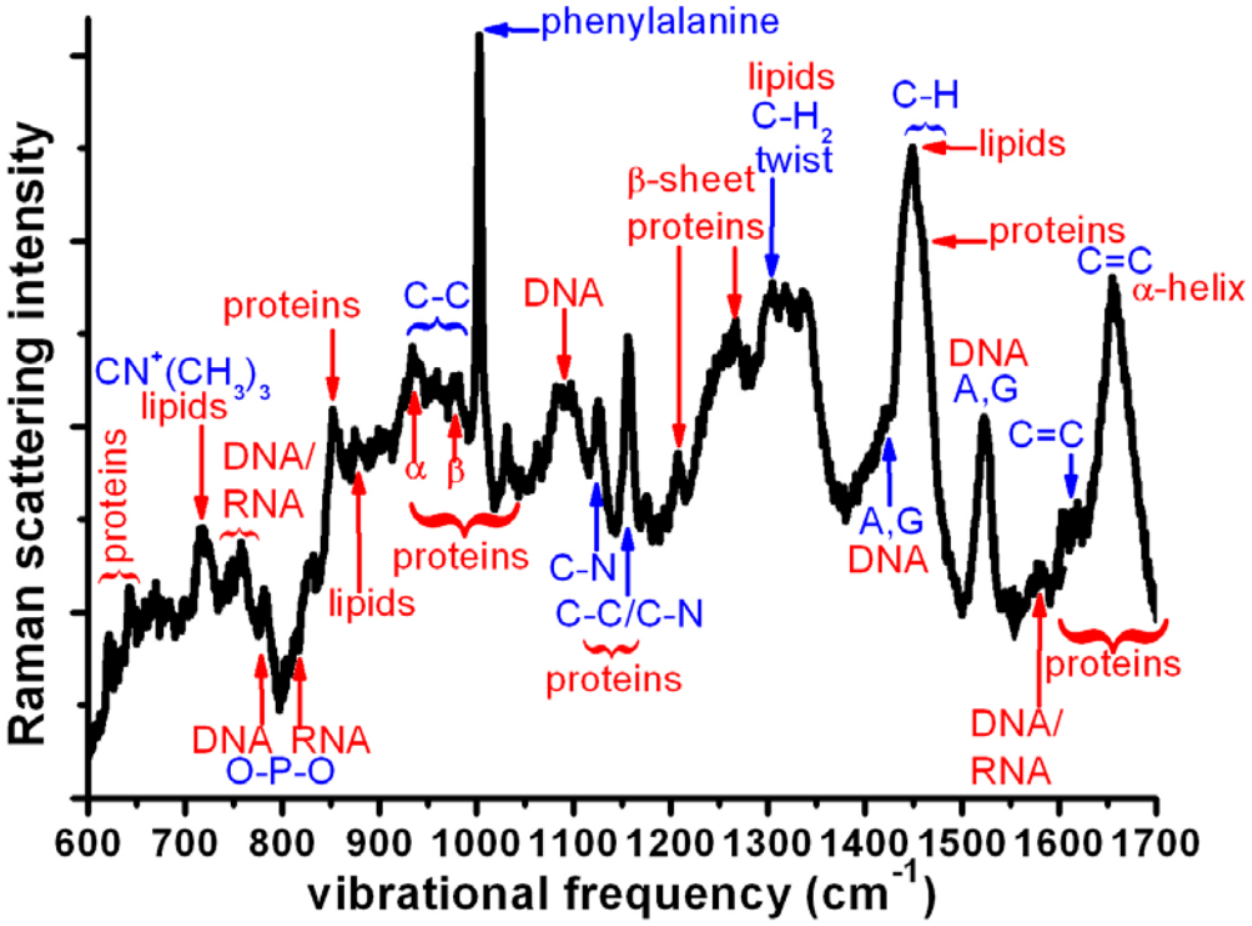

Figure 6.

Figure 6. 'Unprocessed Raman spectrum of live MCF-7 breast cancer cells. 300 seconds acquisition time, 785 nm illumination, approximately 100 mW illumination power.' (Downes A, & Elfick A. Raman Spectroscopy and Related Techniques in Biomedicine. *Sensors* (Basel, Switzerland). 2010. doi:10. 1871-89. 10.3390/s100301871.) Figure 6 highlights the specific bond stretches identified by each peak from wave numbers 600-1700.

Discussion

Comparison between figures 5 and 6 shows similar peaks of interest between 600-1700 cm^{-1}, evidence of which bond stretches identify MCF-7 cells specifically. The spectra represented by figure 5 and passed through PCA/LDA of experiment one and two indicate some active ingredient affecting the MCF-7 cells beyond the alcohol solvent. The identity of this active ingredient remains to be tested by comparing MCF-7 cells dosed with GTX standard and *M. uniflora* extract as the next steps.

Questions about the safety of using *M. uniflora* extract as a medicine are a large motivator for

this study, especially compared to the use of other traditional medicines like Rhododendron honey. While Rhododendron honey will cause people to seek emergency medical care in the event of an overdose, there have been little to no reports of hospitalization or anecdotal stories of hospitalization from people taking the *M. uniflora* extract, as seen on YouTube or as written in homeopathic and naturopathic circles. Another point of safety stems from the fact that *M. uniflora* parasitizes mycelium, which can sequester heavy metals therefore creating a possibility that the mycelium can transfer heavy metals to the aerial parts of *M. uniflora*. This would cause the extract to have unsafe levels of heavy metals. A preliminary analysis using microwave plasma atomic mass emission (MP-AES) tested for the presence of lead in the *M. uniflora* tincture. The MP-AES results found 1,800 ppb of lead in the tincture, 120 times the amount of lead allowed in drinking water. The possible sources of lead in the tincture can lead to an exploration of if or how the mycelium transfers lead, and if the presence of lead was due to where the *M. uniflora* specimens were harvested from.

If *M. uniflora* is confirmed to produce GTX like Rhododendrons, then the safety of taking a neurotoxin which has yet to be thoroughly studied is questionable. Current studies on the efficacy and tissue effect of GTX are animal studies which largely focus on organ specific effects of GTX (27,28,29). Animal studies show cell hemorrhage, shedding, and irregular placement local to GTX injection sites. This leads to significantly higher instances of cell apoptosis for tissue acutely exposed to GTX compared to the control group, with the chronically exposed groups having far higher instances of apoptosis than the acute and control groups (28).

If *M. uniflora* does indeed produce GTX, then there are many people who are taking a risk when consuming the tincture beyond possible heavy metal contamination. The hospitals in the United States of America are not used to seeing patients taking Rhododendron honey, and if more people take *M. uniflora* due to the influence of herbalism books and YouTube videos, then there could be issues with treatments for patients who overdose on *M. uniflora* tincture.

Acknowledgements

This study was funded by the University of Wisconsin – Eau Claire using Blugold Commitment Differential Tuition funds. We would like to thank the Department of Materials Science and Biomedical Engineering for access to equipment used in the study. We would also like to thank Dr. Laurel McEllistrem and Dr. Anthony Wagner for their assistance with the Raman spectroscopy system. A special thanks to Dr. Annabel Renwick and the Sarah P. Duke Gardens for donating Rhododendron leaves to continue this study.

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