Nanopore-based DNA barcoding of herbal supplements by highschoolers expose undeclared plant ingredients

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ABSTRACT

Herbal supplements, also known as herbal medicinal products (HMP) are considered dietary supplements and are not regulated like pharmaceutical drugs by the US Food and Drug Administration (FDA). For the most part, supplement manufacturers are expected to self-regulate, and ensure that their products are safe to be consumed by the public. This has meant that the ingredients listed on supplement bottles are frequently either partially or entirely false and can put consumers' health at risk. In this study, we tested samples of 5 different herbal supplements, each of which claims to contain one or more different plants. We used Nanopore DNA sequencing to help us identify the plants contained within the herbal supplements. We found that all five HMPs contained plants not declared on the label, which make us wary of consuming HMPs. This project also taught us the process of Nanopore sequencing and made us realize how easy, practical, and accessible this technology is for implementation in HMP authentication, that even highschoolers like us can perform.

INTRODUCTION

- Herbal medicinal products (HMPs) are often blends of multiple raw and processed components of plants, such as the roots, stems, seeds, bark, flowers, and leaves (Bent, 2008; Ehrlich, 2015). It's consumed through supplemental pills, powders, teas, and other means for medical purposes or health benefits (Ehrlich, 2015). Dietary supplements are growing in popularity, with supplement sales accounting for an estimated $18.7 billion annually, with herbal supplements accounting for approximately $4.3 billion of that (Institute of Medicine et al., 2005), leading to an increasingly predatory industry, where competitors are able to cut corners without the real threat of repercussions.
- HMPs are consumed by 57.3% of the US population, but the US Food and Drug Administration (FDA) does not regulate this industry, unlike pharmaceutical drugs. Supplement manufacturers are expected to self-regulate and ensure that their products are safe to be consumed by the public. The lack of regulatory oversight of dietary supplements by the FDA has meant that the contents of herbal supplements may be adulterated and/or substituted (Gladwell, 1990; Cohen, 2012; FDA, 2023).
- Studies have found that 59% of HMPs tested contained plant species not listed on the labels, 30 out of 44 contained product substitutes, and only 2 of 12 brands from which products were tested had no substitution, contamination, or fillers within their products (Mishra et al. 202; Newmaster et al., 2013).
- In this study, we used Nanopore-based DNA barcoding because of its accuracy and ability to characterize DNA from multiple species contained within a sample to authenticate plant ingredients in herbal supplements (Ivanova et al., 2016; Wu & Fand Shaw, 2022).

MATERIALS & METHODS

- Five HMPs (Table 1, 1 with a single ingredient and 4 with mixed plant ingredients) in the form of capsules were purchased from an online retailer and from drug stores in New York City.
- Capsules were opened and the contents poured into microfuge tubes. DNA extraction was performed using a modified procedure of the Qiagen DNeasy Plant Mini Kit (Molina et al. 2018). Primers from Cheng et al. (2016: ITS2-u3 and ITS2-u4) were used for PCR amplification, but were tailed to incorporate Oxford Nanopore universal sequences. The protocol for ligation sequencing of amplicons with PCR (SQK-LSK110 with EXP-PBC001) was followed with successfully-amplified samples pooled together. Pooled DNA library was loaded dropwise on the Flongle device.
- Analysis was performed by UBRP students using the Geneious Prime software (version 2023.1.1) from Biomatters Ltd. Raw files were imported, trimmed, and underwent *de novo* assembly to generate consensus sequences.
- Consensus sequences >199 base pairs were compared to the Viridiplantae ITS2 database (Ankenbrand et al. 2015) via BLAST to obtain the top 3 hits. Output contigs with a max sequence length >150 bp were analyzed: if 2/3 of the top hits belonged to the same plant genus, that genus was noted. Using noted contig reads, a pie chart was created to show the percentage of reads for each genus. Genus-level determination was sufficient for our purposes given uncertainties common in plant species circumscriptions.

St. John's wort	*Hypericum perforatum*
Parasite Eliminator	*Artemisia absinthium*, *Syzygium aromaticum*, *Juglans* (black walnut), *Quassia*, *Mentha* x *piperita*, *Dryopteris filix-mas*, *Foeniculum vulgare*, *Viburnum opulus*
Blood sugar support	*Lagerstroemia speciosa,* guggul resin extract *(Commiphora wightii),* bitter melon extract *(Momordica charantia),* licorice root extract *(Glycyrrhiza glabra), Cinnamomum cassia* bark powder, *Gymnema sylvestre,* yarrow flowers *(Achillea millefolium),* cayenne pepper *(Capsicum annuum),* juniper berry extract *(Juniperus),* white mulberry *(Morus alba)* leaf extract
Menopause support	Dong quai *(Angelica sinensis)*, lemon balm extract (*Melissa officinalis)*, red clover (*Trifolium pratense)*, chasteberry *Vitex agnus-castus,* soybean extract (*Glycine max)*, black cohosh root (*Actaea racemosa)*
4-in-1 supplement	*Curcuma longa, Allium sativum, Zingiber officnale*, Black pepper extract (*Piper nigrum)*

Table 1. List of herbal medicinal products (HMP) tested in this study and their labeled ingredients (sample 1 is single-ingredient; rest mixed).

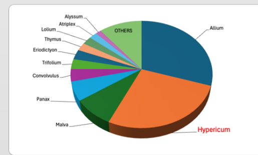

Figure 1. Proportion of sequence reads for all plants detected in HMP marketed as St John's wort (sample 1 in Table 1, single ingredient). Nanopore sequencing confirmed presence of *Hypericum,* as declared on the label, but also detected other plant ingredients not on the label. "OTHERS" refer to contaminating plant genera, each genus with proportion <1% of total.

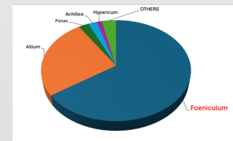

Figure 2. Proportion of sequence reads for all plants detected in HMP marketed as Parasite eliminator (sample 2 in Table 1). Nanopore sequencing confirmed presence of *Foeniculum,* but not the other ingredients on the label *(Artemisia, Syzygium, Juglans, Quassia, Mentha, Dryopteris, Viburnum).* Other plant ingredients not on the label were also detected. "OTHERS" refer to other contaminating plant genera, each genus with proportion <1% of total.

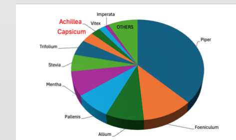

Figure 3. Proportion of sequence reads for all plants detected in HMP marketed as Blood sugar support (sample 3 in Table 1). Nanopore sequencing confirmed presence of *Achillea* and *Capsicum,* but not the other ingredients declared on the label *(Lagerstroemia, Commiphora,, Momordica, Glycyrrhiza, Cinnamomum, Gymnema, Juniperus, Morus).* Other plant ingredients not on the label were also detected. "OTHERS" refer to other contaminating plant genera, each genus with proportion <1% of total.

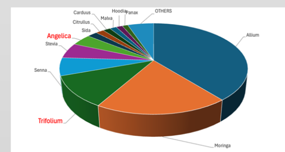

Figure 4. Proportion of sequence reads for all plants detected in HMP marketed as Menopause support (sample 4 in Table 1). Nanopore sequencing confirmed presence of *Angelica* and *Trifolium,* but not the other ingredients declared on the label *(Melissa, Vitex, Glycine, Actaea).* Other plant ingredients not on the label were also detected. "OTHERS" refer to other contaminating plant genera, each genus with proportion <1% of total.

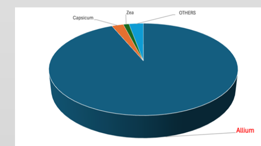

Figure 5. Proportion of sequence reads for all plants detected in HMP marketed as 4-in-1 supplement (sample 5 in Table 1). Nanopore sequencing confirmed presence of *Allium,* but not the other ingredients declared on the label *(Curcuma, Zingiber, Piper*). Other plant ingredients not on the label were also detected. "OTHERS" refer to other contaminating plant genera, each genus with proportion <1% of total.

RESULTS & DISCUSSION

- During the experiment, five herbal medicinal products (HMPs) were processed. One of the samples was a single ingredient HMP, indicated for depression, while the other four contained mixed ingredients with various health claims including the improvement of blood sugar, menopause support, even elimination of parasites.
- For Sample 1, the single ingredient HMP, the sequencing indicated that the sample did contain the ingredient on the bottle, but majority of the DNA identified belonged to species which should not have been in the supplement. For the four mixed samples, while DNA from one or more of the species listed on the bottle was identified, others were missing. The mixed samples each also contained the DNA of plants, which were not included on the label.
- *Allium* was present in all five samples, despite only being listed on one of the product labels. This indicates that the presence of *Allium* was likely due to lab contamination. In order to reduce the risk of contamination, the experiment would have to be redone with fresh reagents.
- There were other plants detected but not declared on the label, which suggests contamination at some point in manufacturing. If it were contamination in the lab, on our part, we'd expect that contaminating plant in all samples, like in the case of *Allium*. Thus, based on our results, consumers are ingesting products they didn't purchase and are lied to by companies, whether deliberately or not.
- While sequencing technology has made evaluation of HMP label accuracy possible, few consumers have access to such equipment, and they may be ingesting potentially harmful plant ingredients. For example, *Sida* and *Apocynum* were detected in the HMPs studied here. *Sida,* specifically *Sida cordifolia*, has been banned because it contains ephedrine, which can cause cardiovascular issues (Cheng et al., 2022). *Apocynum* is highly poisonous and can lead to cardiac arrest (*Apocynum cannabinum*, n.d.). The lack of FDA regulations allows companies to jeopardize customer safety for the sake of profits.
- Unlike Sanger sequencing, which is limited to single-ingredient HMPs, nanopore sequencing is capable of characterizing multiple ingredients, including contaminants, in mixed HMPs. This technology is also easy enough to be used by high school students like us. The findings of this project have heightened our awareness of the risks associated with HMP consumption. Given these concerns, it is recommended that the FDA consider implementing nanopore sequencing for HMP authentication. This could help address the current regulatory gaps and prevent companies from falsely marketing their products.

ACKNOWLEDGEMENTS

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