DNA Barcode Authentication of Devil’s Claw Herbal Dietary Supplements

Genelle L. Diaz-Silveira, Joan Deutsch and Damon P. Little *

Abstract: Devil’s claw is the vernacular name for a genus of medicinal plants that occur in the Kalahari Desert and Namibia Steppes. The genus comprises two distinct species: *Harpagophytum procumbens* and *H. zeyheri*. Although the European pharmacopeia considers the species interchangeable, recent studies have demonstrated that *H. procumbens* and *H. zeyheri* are chemically distinct and should not be treated as the same species. Further, the sale of *H. zeyheri* as an herbal supplement is not legal in the United States. Four markers were tested for their ability to distinguish *H. procumbens* from *H. zeyheri*: *rbcL*, *matK*, *rrnT2*, and *psbA-trnH*. Of these, only *psbA-trnH* was successful. A novel DNA mini-barcode assay that produces a 178-base amplicon in *Harpagophytum* (specificity = 1.00 [95% confidence interval = 0.80–1.00]; sensitivity = 1.00 [95% confidence interval = 0.75–1.00]) was used to estimate mislabeling frequency in a sample of 23 devil’s claw supplements purchased in the United States. PCR amplification failed in 13% of cases. Among the 20 fully-analyzable supplements: *H. procumbens* was not detected in 75%; 25% contained both *H. procumbens* and *H. zeyheri*; none contained only *H. procumbens*. We recommend this novel mini-barcode region as a standard method of quality control in the manufacture of devil’s claw supplements.

Keywords: *Harpagophytum procumbens*; *Harpagophytum zeyheri*; mini-barcode; Pedaliaceae; *psbA-trnH*

1. Introduction

*Harpagophytum* (Pedaliaceae) is a genus of tuberous plants from the Kalahari Desert and Namibia Steppes that is commonly known as devil’s claw due to its hooked fruits [1]. The genus comprises two distinct species—*H. procumbens* and *H. zeyheri*—that have been separated on the basis of morphology [2–4] and chemistry [5]. *Harpagophytum procumbens* consists of two subspecies [3], *H. procumbens* subsp. *procumbens*, which occurs across Namibia, Botswana, and Northern South Africa, and *H. procumbens* subsp. *transvaalense*, which occurs only in the Limpopo region of South Africa. *Harpagophytum zeyheri* comprises three subspecies [3], *H. zeyheri* subsp. *zeyheri*, which is restricted in distribution to northeastern South Africa, and *H. zeyheri* subsp. *schiiffii* and *H. zeyheri* subsp. *sublobatum*, which are both widely distributed across regions of Angola, Zambia, and Zimbabwe and the northern regions of Namibia and Botswana.

There are unsubstantiated reports of possible hybridization in the few places where *H. procumbens* and *H. zeyheri* are sympatric [4,6,7]. Although purporting to demonstrate hybridization, RAPD and ISSR data [7] are, at best, inconclusive: no species-specific genotype groups were detected [7], thus a definitive pattern of hybridization cannot possibly be observed; the published Principal Component Analysis [7]—which is inappropriate for detecting hybridization [8,9]—identifies five putative hybrids, but only one individual is truly intermediate while several non-hybrid samples are equally or more intermediate than the putative hybrids; and the published UPGMA dendrogram [7] refutes the hypothesis of hybridization because it nests the putative hybrids well within the two parental clusters rather than at the cluster base where hybrids are expected to appear [10].
In addition, morphological data [4] purportedly demonstrate hybridization, but they are not statistically significant: the published Discriminant Function Analysis (DFA) [4] improperly implemented DFA such that hybrids were assumed to be present rather than using DFA to test that supposition. In addition, measurements that violate the Gaussian distribution assumed by DFA [11] were included. If DFA is conducted on the five characteristics that do not deviate [12] significantly ($p > 0.01$) from the Gaussian distribution (arm width, seed column height, fruit length, fruit width, and fruit circumference), the putative hybrids [4] are classified without evidence of intermediacy ($pp > 0.99999$). Independent of the improperly implemented DFA, no statistical test was conducted to determine if the putative hybrids were truly intermediate [4]: the character count procedure [9] employing the sign [13] and Scheffé [14] tests ($p = 0.05$) does not indicate intermediacy for any characters and thus no trace of hybridity was detected ($p = 1.0$).

Given this critical review, there are no published data showing evidence of hybridization between *Harpagophytum* species and further study of additional specimens and characteristics is needed to determine if hybridization does indeed occur.

Devil’s claw has traditionally been used to treat dyspepsia, fever, constipation, hypertension, and venereal disease [1]. Commercial preparations of *H. procumbens* are sold to treat arthritis in both the European and United States markets [15]. *Harpagophytum zeyheri* cannot be legally sold as an herbal supplement in the United States [16] but it was appended to the European Pharmacopeia [17]. Both species are wild sourced—primarily from Namibia [18].

Although clinical trials have demonstrated the efficacy of *H. procumbens* for musculo-skeletal pain relief [19–22], animal and in vitro studies have produced conflicting results [23–27]. The suspected active compounds—harpagoside, harpagide, 8-p-coumaroyl-harpagide, and acteoside—inhibit cyclooxygenase (COX) 1 and 2 [28–30] and the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [31,32]. Harpagoside is the main anti-inflammatory agent, but it is less effective in isolation [31] and thus the constituents of *H. procumbens* are thought to have synergistic effects [33].

Commercial herbal supplements are most frequently sold as dry fragments or powders. As a result, the authentication of these materials has traditionally relied upon macro- and microscopic morphological examination along with chemical assays for specific compounds or classes of compounds [34]. In the last two decades, DNA-based assays have become more common with assays for specific plants (e.g., molecular marker-based methods that utilize simple sequence repeats (SSR) or single nucleotide polymorphisms (SNP)) and general untargeted analysis techniques (e.g., short fragment sequencing methods such as whole metagenome analysis and metabarcoding) now being prominently used [35–37]. DNA barcoding has emerged as a preferred method of herbal supplement authentication due to the fact that it generally works well with highly fragmented DNA from high-copy regions (e.g., plastid), can detect multiple species at once, and is relatively inexpensive. These characteristics make the method ideal for assaying the DNA in highly degraded herbal products.

Devil’s claw supplements are sold mainly in capsule or tablet form [38]. Thus, it is impossible to determine which species they contain without additional analysis. A reliable identification method to ensure correct labeling is needed. We aim to create and test a DNA mini-barcode assay for both *Harpagophytum* species.

### 2. Results

#### 2.1. Reference Sequences

Reference sequences from four markers were generated from 39 morphologically identifiable specimens (Table 1). In total, 17 *rbcL*, 23 *matK*, 22 nrITS2, and 35 *psbA-trnH* barcodes were produced. Median sequence quality ($B_{30}$ [39]) exceeds the requirements of the BARCODE data standard (version 2.3 [40]): 0.841 (IQR 0.682–0.936) for *rbcL*, 0.891 (IQR 0.649–0.941) for *matK*, 0.849 (IQR 0.682–0.879) for nrITS2, and 0.845 (IQR = 0.466–0.890) for *psbA-trnH*.
Table 1. Morphologically identifiable reference samples used to generate *rbcL*, *matK*, *nrITS2*, *psbA-trnH* and/or *psbA-trnH* mini-barcode sequences and to validate the *psbA-trnH* mini-barcode. Standard herbarium codes are used [41]. Cultivated specimens are indicated, all others are presumed to be wild collected. All sequences except EU531713 [42] were produced for this study.

| Species                      | Voucher Specimen | Locality                  | Sample Type                           | GenBank Accession |
|------------------------------|------------------|---------------------------|---------------------------------------|-------------------|
| Dicerocaryum zanguebarium    | Loeb and Koch 339 (NY) | Namibia: Oshikango       | reference and validation               | —                 |
| Harpagophytum procumbens     | Allen 308 (MO)   | Botswana: Orapa           | reference and validation               | KT717103          |
| Harpagophytum procumbens     | Davidse and Loxton 6296 (MO) | Namibia: Keetmanshoop        | reference and validation               | KT717109          |
| Harpagophytum procumbens     | de Koning 8142 (MO) | Mozambique: Chigubo        | reference                             | KT717110          |
| Harpagophytum procumbens     | Dinter 396 (MO)  | Namibia: Okahandja        | reference and validation               | —                 |
| Harpagophytum procumbens     | Grignon 239 (MO) | Botswana: Ghanzi          | reference and validation               | KT717128          |
| Harpagophytum procumbens     | Hardy 6575 (MO)  | Namibia: Aranos           | reference and validation               | KT717154          |
| Harpagophytum procumbens     | Herman 1264 (MO) | South Africa: Blouberg Privaatnatuurreservewe | reference and validation               | KT717151          |
| Harpagophytum procumbens     | Lavranos and Bleck 22701 (MO) | Namibia: Otjiwarongo      | reference and validation               | KT717172          |
| Harpagophytum procumbens     | Lavranos and Bleck 22703 (MO) | Namibia: Khorixas         | reference and validation               | KT717147          |
| Harpagophytum procumbens     | Leach 10682 (MO) | Zimbabwe: Beit Bridge     | reference                             | KT717099          |
| Harpagophytum procumbens     | Long and Rae 44 (MO) | Botswana: Jwaneng         | reference and validation               | KT717129          |
| Harpagophytum procumbens     | Ngoni 257 (MO)   | Botswana: Mosu            | reference                             | KT717169          |
| Harpagophytum procumbens     | Owens 19 (MO)    | Botswana: Deception Valley | reference and validation              | KT717169          |
| Harpagophytum procumbens     | Rodin 3539 (NY)  | South Africa: Vryburg     | reference                             | KT717098          |
| Harpagophytum procumbens     | Rogers s.n. (MO) | South Africa: Bellville   | reference                             | KT717119          |
| Harpagophytum procumbens     | Sidey 305 (MO)   | South Africa: Fauresmith  | reference and validation               | KT717143          |
| Harpagophytum procumbens     | Skarpe S-319 (MO) | Botswana: Hukuntsi       | reference and validation               | KT717144          |
| Harpagophytum procumbens     | Smuts and Gillett 2130 (MO) | South Africa: Rooikop     | validation                            | —                 |
Table 1. Cont.

| Species             | Voucher Specimen | Locality                                             | Sample Type                        | GenBank Accession       |
|---------------------|------------------|------------------------------------------------------|------------------------------------|-------------------------|
|                     |                  |                                                      |                                    |                         |
| *Harpagophytum procumbens* | Venter 9637 (MO, NY) | South Africa: Glen Agricultural College               | reference                         | KT717175 KT717106 KT717130 KY706350 |
| *Harpagophytum zeyheri* | Germishuizen 00733 (MO) | South Africa: Bamboeskloof                            | reference and validation           | KT717114 KT717122 KT717159 |
| *Harpagophytum zeyheri* | Germishuizen 990 (MO) | South Africa: Vaalwater                              | reference and validation           | KT717183 — KT717138 KT717160 |
| *Harpagophytum zeyheri* | Luwiika et al. 335 (MO) | Zambia: Lukona Basic School                           | reference                         | KT717116 KT717137 — |
| *Harpagophytum zeyheri* | Mashasha 111 (MO) | Zimbabwe: Victoria Falls                             | reference and validation           | KT717179 KT717111 KT717134 KT717155 |
| *Harpagophytum zeyheri* | Mogg 37171 (MO) | South Africa: Sandsloot                              | reference and validation           | KT717182 KT717113 KT717136 KT717157 |
| *Harpagophytum zeyheri* | Moyo 7 (MO) | Zimbabwe: Victoria Falls                             | reference                         | — KT717118 — |
| *Harpagophytum zeyheri* | Norlindh and Weimarck 5234 (NY) | South Africa: Pietersburg               | reference                         | — — — — KY706353 |
| *Harpagophytum zeyheri* | Rodin 9140 (MO) | Namibia: Rundu                                       | reference and validation           | KT717184 KT717115 KT717121 KT717158 |
| *Harpagophytum zeyheri* | Rushworth 110 (MO) | Zimbabwe: Dina Pan                                  | reference and validation           | KT717180 KT717094 KT717135 KT717156 |
| *Harpagophytum zeyheri* | Yalala 300 (MO) | Botswana: Mahalapye                                 | reference                         | KT717181 KT717112 KT717117 KY706352 |
| *Josephinia euginiae*  | Michell and Boyce 3144 (MO) | Australia: Nitmiluk National Park                    | reference and validation           | — — — — KT717162 |
| *Pedaliodiscus macrocarpus* | Luke et al. TPR 73 (MO) | Kenya: Tana River National Primate Reserve          | reference and validation           | — — — — KT717139 |
| *Pedalium murex*      | Comanor 608 (NY) | Sri Lanka: Potuvil—Panama Road                       | reference and validation           | — — — — KT717140 |
| *Pterodiscus auranthacus* | Seydel 4135 (NY) | Namibia: Windhoek                                   | reference and validation           | — — — — KT717141 |
| *Pterodiscus speciosus* | Zietsman 4079 (NY) | South Africa: Hoopstad                              | reference and validation           | — — — — KT717142 |
| *Rogeria adenophylla* | Seydel 4368 (NY) | Namibia: Windhoek                                   | reference and validation           | — — — — KT717167 |
| *Sesamum indicum*     | Donmez 9932 (NY) | Turkey: Kula                                        | reference and validation           | — — — — KT717164 |
| *Sesamum indicum*     | Nesbitt 1939 (RNG) | —                                                    | reference                         | — — — — EU531713 |
| Species                        | Voucher Specimen | Locality             | Sample Type                  | GenBank Accession |
|-------------------------------|------------------|----------------------|------------------------------|-------------------|
| *Sesamum radiatum*            | Thomas 10563 (NY)| Brazil: Ilhéus       | reference and validation    | —                 |
|                               |                  |                      |                              |                   |
| *Sesamum triphyllum*          | Zietsman and Peyper 4061 (NY) | South Africa: Petrusburg | reference and validation    | —                 |
|                               |                  |                      |                              |                   |
| *Uncarina grandiflora*        | Falk 97001 (NY)  | cultivated           | reference and validation    | —                 |

**Table 1. Cont.**

Within *Harpagophytum*, variation was only observed in psbA-trnH (Figure 1, Figure A1). *Harpagophytum* can be unambiguously distinguished from all other Pedaliaceae by alignment positions 16, 64, and 116. The two *Harpagophytum* species can be differentiated by alignment positions 76 and 107. Intraspecific variation was observed in reference samples of both *H. procumbens* (alignment position 95) and *H. zeyheri* (alignment positions 77 and 88). Only one of these variants is exactly correlated with geography or current taxonomy: position 77 distinguishes *H. zeyheri* subsp. *suboblatum* (sample from Namibia) from *H. zeyheri* subsp. *zeyheri* (samples from South Africa). No samples of *H. zeyheri* subsp. *schiffii* were available for examination.
2.2. Mini-Barcode Validation

Validation psbA-trnH mini-barcode (n = 30) median sequence quality was 0.569 (IQR 0.532–0.587). BRONX [43] was able to correctly identify all H. procumbens validation samples and exclude H. procumbens as a possible identification for all other validation samples (n = 13 H. procumbens; n = 17 other species; specificity = 1.00 [95% confidence interval = 0.80–1.00]; sensitivity = 1.00 [95% confidence interval = 0.75–1.00]; [44]). The absolute consistency of alignment positions 16, 64, 76, 107, and 116 prevent infraspecific variation from having any bearing on Harpagophytum species identification.

2.3. An Analysis of Herbal Supplements

Amplifiable DNA was extracted from 20 of 23 (87%) herbal supplements. Amplification success was significantly correlated with the reports of root extract on product labels (McNemar test [45]; p = 0.04331; Table 2). The failure rate for samples labeled as having root extract (17%) was nearly double that of samples without root extract (9%; Table 2).
Table 2. Herbal dietary supplement label ingredients and *psbA-trnH* mini-barcode determination. Supplement sequence type corresponds to those in Figure 1. If Latin names were not provided on the product label, the Latin name was determined using [16]. Despite being noted on some labels, the sale of supplements containing *H. zeyheri* is not legal in the United States.

| Supplement Sequence Type | Label Species                                                                 | Devil’s Claw Material Type | Contains *H. procumbens* | Contains *H. zeyheri* |
|--------------------------|-------------------------------------------------------------------------------|-----------------------------|--------------------------|-----------------------|
| A                        | *Harpagophytum procumbens, Curcuma longa, Crataegus oxyacantha, Arctium lappa, Smilax febrifuga, Yucca schidigera, Zingiber officinale, and Vaccinium myrtillus* | root extract                | no                       | yes                   |
| A                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| A                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| A                        | Boswellia serrata, Curcuma longa, and *Harpagophytum procumbens*              | root extract                | no                       | yes                   |
| A                        | Boswellia serrata, Uncaria tomentosa, *Harpagophytum procumbens, Yucca schidigera, Gymnema sylvestre, Curcuma longa, Camellia sinensis, and Oryza sativa* | root                        | no                       | yes                   |
| A                        | *Harpagophytum procumbens and Oryza sativa*                                  | root extract                | no                       | yes                   |
| A                        | *Harpagophytum procumbens*                                                    | root extract                | no                       | yes                   |
| A                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| A                        | *Harpagophytum procumbens, Boswellia serrata, Curcuma longa, and Tanacetum parthenium* | root extract                | no                       | yes                   |
| B                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| B                        | *Harpagophytum procumbens*                                                    | root extract                | no                       | yes                   |
| C                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| C                        | *Harpagophytum procumbens and Oryza sativa*                                  | root extract                | no                       | yes                   |
| C                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| C                        | *Harpagophytum procumbens*                                                    | root                        | no                       | yes                   |
| D                        | *Harpagophytum procumbens and/or Harpagophytum zeyheri*                       | root extract                | yes                      | yes                   |
| E                        | *Harpagophytum procumbens*                                                    | root and root extract       | yes                      | yes                   |
| F                        | *Harpagophytum procumbens*                                                    | root                        | yes                      | yes                   |
| F                        | *Harpagophytum procumbens and/or Harpagophytum zeyheri*                       | root extract                | yes                      | yes                   |
| —                        | *Harpagophytum procumbens*                                                    | root                        | unknown                  | unknown               |
| —                        | *Polygonum cuspidatum, Curcuma longa, Zingiber officinale, Camellia sinensis, Harpagophytum procumbens, and Salix alba* | root extract                | unknown                  | unknown               |
| —                        | *Harpagophytum procumbens*                                                    | root extract                | unknown                  | unknown               |

PCR products were successfully sequenced for all 20 amplifiable supplements: mini-barcode median sequence quality was 0.561 (IQR 0.451–0.587)—very similar to the quality of the validation samples.

Harpagophytum zeyheri was found in all 20 fully-analyzable samples: all supplements contained either *H. zeyheri* (75%; 15/20; Types A, B, and C; a “T” at alignment position 76 and a “G” at alignment position 107; Figure 1, Table 2) or a combination of *H. procumbens*. 
and *H. zeyheri* (25%; 5/20; Types D, E, and F; a “K” [“G” and “T”] at alignment positions 76 and 107; Figure 1, Table 2); no supplements contained only *H. procumbens*.

Types A, B, and C contain *H. zeyheri* haplotypes that exhibit the same variation found in the reference samples. Type A is composed of samples that contain only one *H. zeyheri* haplotype, while types B and C are mixtures of *H. zeyheri* haplotypes (e.g., Figure 2). In contrast, types D, E, and F are mixtures of *H. procumbens* and *H. zeyheri* haplotypes. Type E contains one *H. procumbens* haplotype and two *H. zeyheri* haplotypes (a “D” [“A”, “G” and “T”] at alignment position 77; Figure 1).

![Figure 2](image-url)

**Figure 2.** Portions of forward (top) and reverse (bottom) Sanger sequencing chromatograms demonstrating polymorphic positions (alignment positions 77 and 88) in herbal supplement mini-barcode sequences of a Type C sequence. Diagnostic nucleotides (Figure 1) are indicated by their alignment position; “A” = green; “G” = black; “K” = maroon {GT}; “M” = indigo {AC}; “T” = red. Despite the supplement being labeled as containing only *H. procumbens*, alignment position 76 indicates that this sample is composed exclusively of *H. zeyheri*.

3. Discussion

The *psbA-trnH* mini-barcode absolutely differentiates *Harpagophytum* from all other Pedaliaceae (Figure 1: blue highlighted positions 16, 64, and 116) and in turn *H. procumbens* and *H. zeyheri* from one another (Figure 1: orange highlighted positions 76 and 107). Thus, the species have consistent character state differences and can be considered distinct phylogenetic species [46]. The absolute consistency of *psbA-trnH* mini-barcode alignment positions 16, 64, 76, 107, and 116 prevent infraspecific variation from having any bearing on repeatable *Harpagophytum* species identification (specificity = 1.00 [95% confidence interval = 0.80–1.00]; sensitivity = 1.00 [95% confidence interval = 0.75–1.00]). Although there are reports of possible interbreeding between the two *Harpagophytum* species [4,6,7], the pattern observed here is inconsistent with hybridization because the morphological and molecular species identifications exactly match. No intermediate morphological phenotypes have been confirmed either in the literature or in our research, suggesting that hybrids, if they exist, have retained strong morphological similarity to one of the parental species. Therefore, absolute rejection of the hybridization hypothesis would require the investigation of multiple biparentally inherited molecular markers. Given the lack of support for the supposition of hybridization in the data, the regulatory distinction between *H. procumbens* and *H. zeyheri* in the United States [16] can be enforced.
The variation within the \textit{psbA-trnH} mini-barcode used to differentiate between the two \textit{Harpagophyllum} species could be assayed using molecular techniques other than the Sanger sequencing method demonstrated here. For instance, one could use PCR-RFLP with \textit{AseI} (5′-ATTAAAT-3′) to assay alignment position 107 (\textit{H. procumbens} will cut, but \textit{H. zeyheri} will not); RT-PCR with specific primers and/or probes targeted to alignment positions 16, 64, 76, 107, and/or 116; or short read genome skimming (e.g., Illumina) with appropriate bioinformatic postprocessing to find alignment positions 16, 64, 76, 107, and 116 in the output sequences. Depending upon the needs of the user, each of these techniques could be conducted in such a way as to quantify the relative or absolute amounts of DNA from each species present in the sample.

The observed mini-barcode PCR amplification failure rate from herbal supplements of 13% is a bit high compared to the 3–10% reported for similar studies [47–49]. Although the processing of plant materials for herbal supplement manufacturing frequently results in DNA fragmentation and destruction [50–70] that can prevent amplification, the processing techniques used for devil’s claw may be more damaging than those used for other herbal supplements studied thus far—which is supported by the significant correlation between reports of root extract (a relatively damaging technique [70]) on product labels and PCR failure (McNemar test [45]; \( p = 0.04331 \); Table 2). It is also possible that some, or all, of the high rate of PCR failure can be attributed to the amount of recoverable DNA in devil’s claw tap roots being low and/or less enzymatically accessible in comparison to aerial parts as is the case in carrot (\textit{Daucus carota}) tap roots [71,72].

Labels of only two of the 20 analyzable supplements (Table 2) list \textit{Harpagophyllum zeyheri}, but \textit{H. zeyheri} was found in all 20 fully-analyzable samples. Somehow the two, predominantly allopatric [1], species were mixed. Although \textit{H. zeyheri} can be legally sold in the European Union [17], it cannot be sold in the United States [16].

Bulk materials of devil’s claw are usually sold in a morphologically unidentifiable state [1,5]. Thus, a chemical test that measures the relative quantity of harpagoside and 8-p-coumaroyl-harpagide is often used to distinguish between bulk materials from the two species [73]. The data that purport to validate the assay were not analyzed statistically [73]. Unfortunately, the data do not statistically differentiate between the \textit{Harpagophyllum} species (Mann–Whitney test [74]; \( p = 0.1386 \))—perhaps due to the miniscule sample size (\( n = 5 \)). Therefore, this chemical assay cannot be considered reliable. Revalidation with additional, morphologically identifiable and vouchered samples may redeem this assay for harpagoside and 8-p-coumaroyl-harpagide.

Due to the legal status of \textit{H. zeyheri} in the United States, it is imperative that supplement manufacturers employ a robust method of quality control to evaluate all devil’s claw supplements sold. Because the mini-barcode presented here is reliable, cost-efficient, and simple to use, we recommend it as a standard method of quality control instead of the relative quantity of harpagoside and 8-p-coumaroyl-harpagide.

4. Materials and Methods

A barcode reference database of \textit{rbcL}, \textit{matK}, nrITS2, and \textit{psbA-trnH} sequences was created from morphologically identifiable samples of Pedaliaceae. Specimen identifications followed standard references [3,6,75]. Sequences outside \textit{Harpagophyllum} were sampled from close (\textit{Pterodiscus}, \textit{Pedaliodiscus}, \textit{Pedalium}, \textit{Uncaria}, and \textit{Rogeria}) and distant relatives (\textit{Dicerocaryum}, \textit{Josephinia}, and \textit{Sesamum}; Table 1; [76]).

Validation samples were chosen arbitrarily (\( n = 30 \); Table 1). Herbal supplements (capsules and compression tablets) were purchased online.

A \textit{psbA-trnH} mini-barcode was designed from all Pedaliaceae reference sequences. The mini-barcode is anchored within the intergenic spacer (alignment positions 1–122) and extends into \textit{trnH} (alignment positions 123–147; Figure A1). This region was selected for its compactness and discriminatory power.

DNA was isolated [48] from leaves of reference and validation samples and powdered herbal supplements. Markers were amplified using the polymerase chain reaction (PCR).
Each 15 µL reaction contained 1.5 µL PCR buffer (200 mM tris pH 8.8, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% (v/v) Triton X-100, 50% (w/v) sucrose, 0.25% (w/v) cresol red, and 0.25 µg/µL BSA), 0.2 mM of each dNTP, 1.0 µM of each amplification primer, 0.5 units of Taq polymerase, and 0.5 µL DNA. Primer sequences and cycling conditions are given in Tables 3 and 4.

Table 3. PCR primers used for amplification and sequencing.

| Marker           | Primer Name | Sequence (5′→3′) | Source               |
|------------------|-------------|------------------|----------------------|
| matK             | 1R          | ACCCAGTCCATCTGGAAATCTTGGTTC | K.J. Kim (pers. com.) |
| matK             | 3F          | CGTACAGTACTITTGTGTACACAG | K.J. Kim (pers. com.) |
| nrITS2           | S2F         | ATGCAGTACCTTGTTGACATC | [77]                 |
| nrITS2           | S3R         | GACGCTTCTCCAGACTACAAT | [77]                 |
| psbA-trnH        | psbAF       | GTTATGCATGAACTGAACTGC | [78]                 |
| psbA-trnH        | trnHR       | CGCGCATGGTGGATTCACAAATC | [78]                 |
| psbA-trnH        | mini-barcode F | GAAGATAAATGAAATGATTGAAATGC | novel               |
| psbA-trnH        | mini-barcode R | TGGATTCGAAATGCATGACAAAT | [78]                 |
| rbcL             | 32F         | TTGGATTCAAAGCTGGTGTT | [79]                 |
| rbcL             | a_F         | ATGTCACCACAAACAGACTAAAGC | [80]                 |
| rbcL             | ajf634R     | GAAACGCTGTCTTCCAACACGCT | [81]                 |

Table 4. PCR cycling conditions used. Amplification reactions used an initial denaturation of 150 s at 95 °C and a final extension of 600 s at 72 °C (psbA-trnH used 64 °C). Primer names correspond to those in Table 3.

| Marker           | Primers | Cycling                                                        |
|------------------|---------|----------------------------------------------------------------|
| matK             | 1R & 3F | 10 × (30 s, 95 °C; 30 s, 56 °C; 30 s, 72 °C); 25 × (30 s, 88 °C; 30 s, 56 °C; 30 s, 72 °C) |
| nrITS2           | S2F & S3R | 35 × (30 s, 95 °C; 30 s, 56 °C; 30 s, 72 °C) |
| psbA-trnH        | psbAF & trnHR | 10 × (30 s, 95 °C; 120 s, 55 °C); 23 × (45 s, 90 °C; 120 s, 55 °C) |
| psbA-trnH        | F & R    | 35 × (30 s, 95 °C; 120 s, 58 °C) |
| rbcL             | 32F & ajf634R | 35 × (30 s, 95 °C; 30 s, 58 °C; 30 s, 72 °C) |
| rbcL             | a_F & ajf634R | 35 × (30 s, 95 °C; 30 s, 58 °C; 30 s, 72 °C) |

PCR products were treated with ExoSapIt (ThermoFisher, Waltham, MA), and sequenced bidirectionally on a 3730 automated sequencer (ThermoFisher) using the amplification primers and BigDye 3.1 (ThermoFisher).

KB 1.4 (ThermoFisher) was used to generate base calls and quantity values from raw chromatograms. Contigs were assembled and edited with Sequencher (version 5.2.3; Gene Codes, Ann Arbor, MI). Sequence quality was determined using B (version 1.2; [39]) with expected coverage (x) set to the number of reads. Newly generated mini-barcode sequences were compared to reference sequences using BRONX (version 2.0; [43]). R version 3.3.1 (http://www.R-project.org, accessed on 21 August 2021) was used to calculate discriminant function analysis [11], the Mann–Whitney test [74], the McNemar test [45], the Scheffé [14] test, the Shapiro–Wilk test [12], the sign test [13], and specificity and sensitivity [44].

Author Contributions: Conceptualization, D.P.L.; methodology, G.L.D.-S. and J.D.; writing—original draft preparation, G.L.D.-S. and D.P.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding;
Institutional Review Board Statement: Not applicable.
Informed Consent Statement: Not applicable.
Data Availability Statement: Sequences were deposited in GenBank accessions KT717094–KT717184 and KY706348–KY706353.

Acknowledgments: We thank Christian Schulz for translating reference material from the German.
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Figure A1. Full psbA-trnH mini-barcode alignment.

References
1. Stewart, K.M.; Cole, D. The commercial harvest of Devil’s Claw (Harpagophyton spp.) in Southern Africa: The Devil’s in the details. *J. Ethnopharmacol.* 2005, 100, 225–236. [CrossRef]
2. Decaisne, J. Revue du groupe des pedalinees. *Ann. Sci. Nat. Bot.* 1865, 5, 321–336.
3. Ihlenfeldt, H.-D.; Hartmann, H. Die gattung *Harpagophyton* (Burch.) DC. Ex Meissn. (Monographie Der Afrikanischen Pedaliaceae II). *Mitt. Aus Dem Staatsinst. Fur Allg. Bot. Hambg.* 1970, 13, 15–69.
4. Muzila, M.; Setshogo, M.P.; Mpoloka, S.W. Multivariate Analysis of *Harpagophyton* DC. Ex Meisn (Pedaliaceae) based on fruit characters. *Int. J. Biodivers. Conserv.* 2011, 3, 101–109.
5. Mncwango, N.P.; Viljoen, A.M.; Zhao, J.; Vermaak, I.; Chen, W.; Khan, I. What the devil is in your phytomedicine? Exploring species substitution in *Harpagophyton* through chemometric modeling of 1H-NMR and UHPLC-MS datasets. *Phytochemistry* 2014, 106, 104–115. [CrossRef] [PubMed]
6. Ihlenfeldt, H.-D. Pedaliaceae. In *Flora Zambesiaca*; Royal Botanic Gardens Kew: Richmond, Surrey, UK, 1988; Volume 8, pp. 86–113.
7. Muzila, M.; Werlemark, G.; Ortiz, R.; Sefic, J.; Fatih, M.; Setshogo, M.; Mpoloka, W.; Nybom, H. Assessment of diversity in *Harpagophyton* with RAPD and ISSR markers provides evidence of introgression. *Hereditas* 2014, 151, 91–101. [CrossRef]
10. McDade, L.A. Hybrids and phylogenetic系统atics III. Comparison with distance methods. *Syst. Bot.* 1997, 22, 669–683. [CrossRef]

11. Fisher, R.A. The use of multiple measurements in taxonomic problems. *Ann. Eugen.* 1936, 7, 179–188. [CrossRef]

12. Shapiro, S.S.; Wilk, M.B. An analysis of variance test for normality (complete samples). *Biometrika* 1965, 52, 591–611. [CrossRef]

13. Arbuthnott, J. An argument for divine providence, taken from the constant regularity observed in the births of both sexes. *Philos. Trans.* 1710, 27, 186–190.

14. Scheffé, H. A method for judging all contrasts in the analysis of variance. *Biometrika* 1953, 40, 87–104.

15. Marshall, N.T. *Searching for a Cure: Conservation of Medicinal Wildlife Resources in East and Southern Africa*; Traffic International: Cambridge, UK, 1998.

16. McGuffin, M.; Kartesz, J.T.; Leung, A.Y.; Tucker, A.O. *Herbs of Commerce*, 2nd ed. American Herbal Products Association: Silver Spring, MD, USA, 2000.

17. Grote, K. *The Increased Harvest and Trade of Devil’s Claw (Harpagophytum procumbens) and Its Impacts on the Peoples and Environment of Namibia, Botswana and South Africa*; Global Facilitation Unit for Underutilized Species: Maccarese, Italy, 2003.

18. Raimondo, D.; Donaldson, J. *The Trade, Management and Biological Status of Harpagophytum Spp. in Southern African Range States*; Convention on International Trade in Endangered Species of Wild Fauna and Flora: Geneva, Switzerland, 2002.

19. Chantre, P.; Cappelaere, A.; Leblan, D.; Guedon, D.; Vandermander, J.; Fournie, B. Efficacy and tolerance of *Harpagophytum procumbens* versus diclofenac in the treatment of osteoarthritis. *Phytotherapy* 2000, 7, 177–183. [CrossRef]

20. Chrubasik, S.; Thanner, J.; Künzel, O.; Conradt, C.; Black, A.; Pollak, S. Comparison of outcome measures during treatment with the proprietary *Harpagophytum* extract Doloteflin® in patients with pain in the lower back, knee or hip. *Phytotherapy* 2002, 9, 181–194. [CrossRef]

21. Frerick, H.; Schmidt, U. Stufenschema bei coxarthrose. *Der Kassenarzt* 2001, 5, 34–41.

22. Wegener, T.; Lüpké, N. Treatment of patients with arthrosis of hip or knee with an aqueous extract of Devil’s Claw (*Harpagophytum procumbens* DC.). *Phytother. Res.* 2003, 17, 1165–1172. [CrossRef] [PubMed]

23. Andersen, M.L.; Santos, E.H.R.; Maria de Lourdes, V.S.; da Silva, A.A.B.; Tufik, S. Evaluation of acute and chronic treatments with *Harpagophytum procumbens* on Freund’s adjuvant–induced arthritis in rats. *J. Ethnopharmacol.* 2004, 91, 325–330. [CrossRef]

24. Baghdikian, B.; Lanher, M.C.; Fleurentin, J.; Ollivier, E.; Maillard, C.; Balansard, G.; Mortier, F. An analytical study and anti–inflammatory and analgesic effects of *Harpagophytum procumbens* and *Harpagophytum zeyheri*. *Planta Med.* 2007, 63, 171–176. [CrossRef] [PubMed]

25. Kundu, J.K.; Mossanda, K.S.; Na, H.-K.; Suth, Y.-J. Inhibitory effects of the extracts of *Sutherlandia frutescens* (L.) R. Br. and *Harpagophytum procumbens* DC. on phorbol ester–Induced COX-2 expression in mouse skin: AP-1 and CREB as potential upstream targets. *Cancer Lett.* 2005, 218, 21–31. [CrossRef]

26. McLeod, D.W.; Revell, P.; Robinson, B.V. Investigations of *Harpagophytum procumbens* (Devil’s Claw) in the treatment of experimental inflammation and arthritis in the rat. *Br. J. Pharmacol.* 1979, 66, 140P–141P.

27. Whitehouse, L.W.; Znamirovska, M.; Paul, C.J. Devil’s Claw (*Harpagophytum procumbens*): No evidence for anti–inflammatory activity in the treatment of arthritic disease. *Can. Med. Assoc. J.* 1983, 129, 249–251.

28. Abdelouahab, N.; Heard, C. Effect of the major glycosides of *Harpagophytum procumbens* (Devil’s Claw) on epidermal cyclooxygenase-2 (COX-2). *Vitro Integr. Pharmacol.* 2008, 71, 746–749. [CrossRef] [PubMed]

29. Gyrkovska, V.; Alipieva, K.; Maciuk, A.; Dimitrova, P.; Ivanovska, N.; Haas, C.; Bley, T.; Georgiev, M. Anti–inflammatory activity of Devil’s Claw in vitro systems and their active constituents. *Food Chem.* 2011, 125, 171–178. [CrossRef]

30. Jang, M.-H.; Lim, S.; Han, S.-M.; Park, H.-J.; Shin, I.; Kim, J.-W.; Kim, N.-J.; Lee, J.-S.; Kim, K.-A.; Kim, C.-J. *Harpagophytum procumbens* suppresses lipopolysaccharide–stimulated expressions of cyclooxygenase-2 and inducible nitric oxide synthase in fibroblast cell line L929. *J. Pharmacol. Sci.* 2003, 93, 367–371. [CrossRef]

31. Fieberich, B.L.; Heinrich, M.; Hiller, K.O.; Kammerer, N. Inhibition of TNF-α synthesis in LPS–stimulated primary human monocytes by *Harpagophytum* extract SteiHap 69. *Phytotherapy* 2001, 8, 28–30. [CrossRef] [PubMed]

32. Inaba, K.; Murata, K.; Naruto, S.; Matsuda, H. Inhibitory effects of Devil’s Claw (secondary root of *Harpagophytum procumbens*) extract and harpagoside on cytokine production in mouse macrophages. *J. Nat. Med.* 2010, 64, 219–222. [CrossRef]

33. Georgiev, M.I.; Ivanovska, N.; Alipieva, K.; Dimitrova, P.; Verpoorte, R. Harpagoside: From Kalahari Desert to pharmacy shelf. *Phytochemistry* 2013, 92, 8–15. [CrossRef]

34. Ichinomiya, T.; Araya, T.; Kudoh, M. A method for judging all contrasts in the analysis of variance. *Biometrika* 1953, 40, 87–104.

35. Marshall, N.T. *Searching for a Cure: Conservation of Medicinal Wildlife Resources in East and Southern Africa*; Traffic International: Cambridge, UK, 1998.
67. Bergerová, E.; Hrnčirová, Z.; Stankovska, M.; Lopasovska, M.; Siekel, P. Effect of thermal treatment on the amplification and quantification of transgenic and non–transgenic soybean and maize DNA. *Food Anal. Methods* 2010, 3, 211–218. [CrossRef]

68. Costa, J.; Mafra, I.; Amaral, J.S.; Oliveira, M.B.P.P. Monitoring genetically modified soybean along the industrial soybean oil extraction and refining processes by polymerase chain reaction techniques. *Food Res. Int.* 2010, 43, 301–306. [CrossRef]

69. Särkinen, T.; Staats, M.; Richardson, J.E.; Cowan, R.S.; Bakker, F.T. How to open the treasure chest? Optimising DNA extraction from herbarium specimens. *PLoS ONE* 2012, 7, e43808. [CrossRef] [PubMed]

70. Lu, Z.; Rubinsky, M.; Babajanian, S.; Zhang, Y.; Chang, P.; Swanson, G. Visualization of DNA in highly processed botanical materials. *Food Chem.* 2018, 245, 1042–1051. [CrossRef] [PubMed]

71. Boiteux, L.S.; Fonseca, M.E.N.; Simon, P.W. Effects of plant tissue and DNA purification method on randomly amplified polymorphic DNA-based genetic fingerprinting analysis in carrot. *J. Am. Soc. Hortic. Sci.* 1999, 124, 32–38. [CrossRef]

72. Bowman, M.J.; Simon, P.W. Quantification of the relative abundance of plastome to nuclear genome in leaf and root tissues of carrot (*Daucus carota*, L.) using quantitative PCR. *Plant. Mol. Biol. Rep.* 2013, 31, 1040–1047. [CrossRef]

73. Schmidt, A.H. Validation of a fast–HPLC method for the separation of iridoid glycosides to distinguish between the *Harpagophyllum* species. *J. Liq. Chromatogr. Relat. Technol.* 2005, 28, 2339–2347. [CrossRef]

74. Mann, H.B.; Whitney, D.R. On a Test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Stat.* 1947, 18, 50–60. [CrossRef]

75. Ihlenfeldt, H.-D. Pedaliaceae. In *The Families and Genera of Vascular Plants VII: Flowering Plants; Dicotyledons: Lamiales (except Acanthaceae including Avicenniaceae)*; Kadereit, J.W., Ed.; Springer: Berlin, Germany, 2004; pp. 307–322.

76. Gormley, I.C.; Bedigian, D.; Olmstead, R.G. Phylogeny of Pedaliaceae and Martyniaceae and the placement of *Trapella* in Plantaginaceae s. l. *Syst. Bot.* 2015, 40, 259–268. [CrossRef]

77. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* 2010, 5, e8613. [CrossRef]

78. Sang, T.; Crawford, D.J.; Stuessy, T.F. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *Am. J. Bot.* 1997, 84, 1120–1136. [CrossRef]

79. Lledo, M.D.; Crespo, M.B.; Cameron, K.M.; Fay, M.F.; Chase, M.W. Systematics of Plumbaginaceae based upon cladistic analysis of rbcL sequence data. *Syst. Bot.* 1998, 23, 21–29. [CrossRef]

80. Levin, R.A.; Wagner, M.B.; Hoch, P.C.; Nepokroeff, M.; Pires, J.C.; Zimmer, E.A.; Sytsma, K.J. Family–level relationships of Onagraceae based on chloroplast rbcL and ndhF data. *Am. J. Bot.* 2003, 90, 107–115. [CrossRef] [PubMed]

81. Fazekas, A.J.; Burgess, K.S.; Kesnakurti, P.R.; Graham, S.W.; Newmaster, S.G.; Husband, B.C.; Percy, D.M.; Hajibabaei, M.; Barrett, S.C.H. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS ONE* 2008, 3, e2802. [CrossRef] [PubMed]