A novel nucleic acid extraction method from aromatic herbs and dried herbal powders using cow skim milk

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Authenticity of dried aromatic herbs and herbal powders for the ASU (ayurvedic, siddha, unani) drug formulations is a key of their clinical success. The DNA based authentication is an answer; however, extraction of PCR quality DNA from such material is often problematic due to the presence of various co-extracted PCR inhibitors. Here, we report a novel DNA isolation and purification method utilizing cow skim milk that successfully yields PCR quality DNA from the aromatic herbs and dried herbal powders. The improved method presented in this study could be used as an alternative to successfully extract PCR quality DNA from such plant materials. Further, we present a set of robust matK primers which could be used as plant barcoding resource in future studies.

The medicinal plant based formulations have long been used for the treatment of various human ailments since ancient times till modern days. The unsustainable use of these plants has created a negative pressure on their biodiversity and availability; which also leads to unethical malpractices in the current times by the practitioners and traders of the medicinal plants. During recent years, there have also been reports pertaining to the toxicity of various plant based formulations. Such complaints, however, are also being attributed to a larger extent with the unethical malpractices such as mislabeling, fraudulently replacement as well as unintentional negligence. Since most of the times the real identity of the plant remains spurious; therefore, unintended plant formulation is given to the patient, leading to toxicity. Thus, the correct identification and authentication of medicinal plants for their safe use is the need of the hour and seeks global attention.

DNA barcoding has emerged as a modern reliable tool for the identification and authentication of individual medicinal and herbal plants at molecular level. This however, requires extraction of PCR quality DNA from such material. Extraction of DNA from herbal and aromatic plants is often problematic, because these plants contain high levels of secondary metabolites including lipids, phenolic compounds, and viscous polysaccharides that can interfere with downstream molecular applications. The problem becomes even worse if the material under investigation is dried herb or herbal powder, since the quantity and purity of DNA often recovered from such material is not up to the mark, leading to the failure of downstream molecular applications. Thus, the DNA isolation from such herbal materials so far heavily depends on expensive commercial kits.

Here, we have developed a novel method for the extraction of PCR quality DNA from aromatic herbs and dried herbal powders, without the use of any expensive commercial DNA extraction kit. Our method is modified from the cetyltrimethylammonium bromide (CTAB) method and the key novel step in this method is the use of cow skim milk (0.1% fat) during the CTAB lysis of dried herbs and aromatic plants. Skim milk possibly acts by adsorbing the DNA and competing with other adsorption competitors and impurities present in the crude lysates. In the second stage of our procedure, the skim milk adsorbed DNA is purified and separated from co-extracted impurities using routine phenol:chloroform extraction.

Material and methods

The plant material. Various dried herbs, which often include the dried seed, stem part, flowers or any other part of various herbal plants, are being sold in the online marketplace in India. Since these herbs are dried, these are often stored in shops and other marketplace at room temperature. We ordered small quantities of a total of 18...
In order to assess the presence of contaminating DNA from the skim milk, the DNA obtained from the untreated and skim milk treated plant samples were amplified using animal specific universal primers mcb398 and mcb869 previously developed by us19 to generate the species-specific molecular signature (Supplementary Fig. 5). The amplification was carried out in a 20 μl reaction volume containing 10 μl of 2 × Emerald Taq PCR mix (Takara, USA), 5 pM of each primer (mcb398 and mcb869), 7 μl miliQ water, and 1.0 μl template DNA (30–50 ng). The PCR conditions were: an initial denaturation at 95 °C for 5 min, followed by 35 cycles each of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. After amplification, the PCR products were resolved in 2% agarose gel to determine the band profile and the molecular size of the PCR amplicons.
| S.N | Sample code | Dried herb name as on label / voucher code | NCBI accession no.* | Highest bits | Query cover % | BLAST E value | % Nucleotide Similarity | NCBI accession no.** | Herb identity revealed as |
|-----|-------------|------------------------------------------|---------------------|--------------|--------------|--------------|------------------------|---------------------|-----------------------------|
| 1   | H7          | Dashmool Kwath†—Dashmool Bharad (CCMB:29–122:H7) | MN006741            | 1,321        | 100          | 0.0          | 100                    | MF694887.1          | Tribulus sp. CCMB H7*     |
| 2   | H8          | Adulsa—Justicia adhatoda (CCMB:29–123:H8) | MN006742            | 1,317        | 100          | 0.0          | 99.86                  | MG947002.1          | Justicia adhatoda/ Adhatoda vasica* |
| 3   | H12         | Beejband††—Sida cordifolia (CCMB:29–128:H12.1) | MN006743            | 1,327        | 100          | 0.0          | 100                    | KY952501.1          | Rumex sp. CCMB H12       |
| 4   | H13         | Balantshepa Dill Seed/Savaa Seed/Anethum graveolens Seed (CCMB:29–131:H13.2) | MN006744            | 1,267        | 100          | 0.0          | 100                    | MG946951.1          | Anethum graveolens       |
| 5   | H14         | Lendi Pipali—Piper longum seed (CCMB:30–134:H14) | MN006745            | 1,360        | 100          | 0.0          | 99.73                  | MH287271.1          | Piper longum*             |
| 6   | H19         | Godmar—Gymnema sylvestre (CCMB:30–135:H19) | MN006746            | 1,391        | 100          | 0.0          | 100                    | KX911179.1          | Gymnema sylvestre        |
| 7   | H21         | Lajvanti Bee††—Mimosa pudica Seed (CCMB:29–129:H21) | MN006747            | 1,303        | 100          | 0.0          | 99.17                  | GU135078.1          | Hygrophila polysperma     |
| 8   | H24         | Indrajav—Holarrhena pubescens (CCMB:29–130:H24) | MN006748            | 1,371        | 100          | 0.0          | 99.87                  | EF456271.1          | Holarrhena pubescens     |
| 9   | H35         | Kantkari/Ringani—Solamum suatense (CCMB:30–136:H35) | MN006749            | 1,291        | 100          | 0.0          | 100                    | MH085988.1          | Solanum sp. CCMB H35    |
| 10  | H39         | Kamal Beej/Kamal Gatta—Lotus Seed (CCMB:33–144:H39) | MN006750            | 1,295        | 100          | 0.0          | 100                    | LC438879.1          | Nelumbo sp. CCMB H39     |
| 11  | H43         | Sagar Gotti/Latakaran—Molucca Bean/Caesalpinia bonduc (CCMB:33–145:H43) | MN006751            | 1,365        | 100          | 0.0          | 99.87                  | LC080892.1          | Caesalpinia sp. CCMB H43 |
| 12  | H49         | Bavchi—Poralea coryifoila (CCMB:30–139:H49) | MN006752            | 1,387        | 100          | 0.0          | 100                    | MK069582.1          | Calliandra coryifoila/ Poralea coryifoila* |
| 13  | H54         | Kulahi—Dolichos biflorus (CCMB:30–137:H54) | MN006753            | 1,376        | 100          | 0.0          | 100                    | EU717410.1          | Macrotyloma sp./ Dolichos biflorus* |
| 14  | H56         | Kavach Beej Black—Mucuna pruriens Seed (CCMB:33–142:H56) | MN006754            | 1,315        | 100          | 0.0          | 99.72                  | KF621103.1          | Mucuna cochinchinensis/Mucuna pruriens* |
| 15  | H59         | Paneer Phul—Withania coagulans (CCMB:30–133:H59) | MN006755            | 1,371        | 100          | 0.0          | 100                    | MG947039.1          | Withania sp. CCMB H59   |
| 16  | H60         | Palas Beej—Butea monosperma Seed (CCMB:34–241:H60) | MN006756            | 970          | 100          | 0.0          | 100                    | KY628018.1          | Butea monosperma*        |
Table 2. Various dried herbs included in this study to validate the DNA extraction procedure and the success of downstream molecular analyses. Photographs of each of these dried herbs are provided in Fig. 1. *NCBI Accession Nos. of the novel sequences generated in this study. **NCBI Accession No. of the best BLAST hit with the corresponding sequences in NCBI database. †The highest BLAST score of the matK signature sequence obtained from this sample was with that of the signature sequence of Tribulus sp. NCBI accession no. MF694887.1 indicating its identity as that of the Tribulus sp. The identity of this specimen was confirmed by inclusion of reference samples for Tribulus sp. (Reference code WB35—Supplementary Fig. 3, Supplementary Table 5). This also indicated that the taxonomic fidelity of the matK sequences generated in the current study could be considered as high. 2,3,4,7 The presence of these specimen vouchers of dried herbs was also confirmed by inclusion of reference samples for Adhatoda vasica/Justicia adhatoda2 (synonyms), Piper longum3, Psoralea sp./Callen sp.4 (synonyms), and Butea monosperma5, respectively (Reference code CH17, CH10, SV3/J and WB4 respectively in Supplementary Fig. 3). The amplified matK sequences from these reference samples were comparable to that of the test herb (Supplementary Table 5). 5Dolichos and Macrotyloma genus are synonyms. 6Mucuna pruriens and Mucuna cochinchinesis are synonyms. 7According to standard text of Ayurveda, 'Dashmool' is the name given to 10 roots of certain plants that include Bilva root (Aegle marmelos), Agnimantha root (Premania integrifolia), Shyonaka root (Oroxylum indicum), Patula root (Sterospermum suaveolens), Kayangi root (Gmelina arborea), Bruhati root (Solania indicum), Kantakari root (Solania xanthocarpum), Shalaparni root (Desmodium gangeticum), Prushniparni root (Uraria picta), and Gokshura root (Tribulus terrestris). The herb 'Dashmool Bharad' is sold in online marketplace without the clue of its scientific name. A search in Pubmed using keyword "Dashmool Bharad" also finds only one reference about it (J Ayurveda Integr Med, 2015, S26–S32. PubMed Central PMCID: PMC4456680), wherein, in the in vitro evaluation of Anti-oxidant and Anti-inflammatory activities of Dashmool Bharad has been done. However, this reference also does not mention the scientific identity of 'Dashmool Bharad.' Our study revealed that the 'Dashmool Bharad' is indeed the plant part of Tribulus sp., one of the 10 components of 'Dashmool.' 10Identity of these dried herbs was found to be spurious either due to mislabeling or fraudulently replacement, indicating the incidences of malpractices in online herbal marketplace.

51 °C for 1 min, and extension at 72 °C for 2 min. The extension step at the 35th cycle was held for 10 min. The 5 μl of PCR amplicons were loaded in 1% agarose gel to check the PCR success. The PCR products obtained were sequenced using Sanger sequencing in 3730XI DNA Analyser (Thermo Fisher Scientific, USA) on both strands in duplicate and the sequence resolved were blasted against nr databases of NCBI using BLAST program and the ID of the species of contaminating DNA from skim milk was established using the procedure as described before.

Modified matK primers, PCR amplification and sequencing. The matK primers used in this study were modified from Jing et al.28 to give a robust amplification from a wide range of plant taxa. For this, more than 20 thousand available full and partial matK gene sequences were obtained from GenBank database of National Center for Biotechnology Information and aligned using ClustalX2 programme31 (data not shown). The aligned sequences were opened in BioEdit Sequence Alignment Editor V7.2.532 and manually searched for the highly conserved regions to develop universal degenerate primers. The final sequence of the primers used in this study was—matK1472F: 5'-CCCTYTACATCTGGAAATCTTGTTC-3' and CCMB-matK1248R: 5'-GCTRTRTAATGAGAAGATTCTGC-3'. The PCR reactions were conducted in a 20 μl mixture system containing 10 μl 2× Emerald Taq PCR mix (Takara, USA), 5 pM of each primer, 7 μl miliQ water, and 1.0 μl template DNA (30–50 ng). The PCR conditions were: an initial denaturation at 95 °C for 5 min, followed by 35 cycles each of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min. The final extension at 72 °C was held for 10 min. The PCR products obtained were sequenced using Sanger sequencing in 3730XI DNA Analyser (Thermo Fisher Scientific, USA) on both strands in duplicate and the sequence resolved were blasted against nr databases of NCBI using BLAST program23 and the ID of the plant material was established using the procedure as described before.

Results and discussion
DNA isolation of herbal material can be a challenge due to inhibitors that result in sub-optimal DNA quality. This study takes a look at using skimmed-milk to enhancing the standard CTAB protocol in extracting DNA from herbal material. Here, we have shown that our modified CTAB extraction protocol using skim milk was able to generate quality DNA to successfully amplify PCR products. In addition, due to the issues surrounding
Figure 1. Dried herbs included in this study. Refer to Table 2 for the details.
the difficulty in the identification of herbal plant, we created our own primers to determine whether the herb materials used in the study have been correctly identified.

Skim milk is reported to prevent the degradation of DNA and its adsorption by soil colloids17,24. Skim milk was also found effective in extraction of DNA from recalcitrant soil that strongly adsorb DNA, indicating that it acts as the adsorbent competitors present in the crude lysates25. Taking the clue from above studies, we anticipated that the skim milk might also help in the extraction of pure DNA from dried aromatic herbs and herbal powders, where it is difficult to extract pure DNA due to the presence various secondary metabolites that inhibit the PCR reactions and hamper the downstream molecular applications. Our assumption was indeed found true, which was tested and validated in this study. The DNA was isolated from 12 randomly selected dried herbs (see “Methods”, Table 1, Fig. 1) with or without addition of skim milk in the first step of DNA isolation as described in Methods section. The DNA obtained from both sets of experiments was subjected to PCR amplification using modified matK primers developed in this study (see material and methods section). The PCR band obtained from untreated samples are shown in panel ‘b’; and the PCR band obtained from skim milk treated samples are shown in panel ‘d’. The lanes marked as ‘+ve’ in panels ‘b’ and ‘d’ are the ‘positive’ control for PCR and the lanes marked as ‘–ve’ are the ‘internal negative control’ for PCR reactions. The molecular weight markers (Lane M) used are Thermo Scientific GeneRuler® 1 kb DNA Ladder (panel ‘a’ and ‘c’) and Thermo Scientific GeneRuler™ 100 bp Plus DNA Ladder (panel ‘b’ and ‘d’). The uncropped, multiple original exposures of the full length agarose gels displayed here are shown in Supplementary Fig. 4a–d.

Figure 2. Agarose gel images showing the patterns of isolated DNA and amplified PCR products obtained from dried herbs included in this study. The DNA was isolated from 12 randomly selected dried herbs (Table 1) without (a) or with (c) addition of skim milk in the first step of DNA isolation as described in Methods section. The DNA obtained from both sets of experiments was subjected to PCR amplification using modified matK primers developed in this study (see material and methods section). The PCR band obtained from untreated samples are shown in panel ‘b’; and the PCR band obtained from skim milk treated samples are shown in panel ‘d’. The lanes marked as ‘+ve’ in panels ‘b’ and ‘d’ are the ‘positive’ control for PCR and the lanes marked as ‘–ve’ are the ‘internal negative control’ for PCR reactions. The molecular weight markers (Lane M) used are Thermo Scientific GeneRuler® 1 kb DNA Ladder (panel ‘a’ and ‘c’) and Thermo Scientific GeneRuler™ 100 bp Plus DNA Ladder (panel ‘b’ and ‘d’). The uncropped, multiple original exposures of the full length agarose gels displayed here are shown in Supplementary Fig. 4a–d.

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which are being used in various systems of plants based medicines around the world. There is an urgent need for a global effort to develop a high fidelity reference database of authentic, dried medicinal herbs. Therefore, we also noticed the instances of malpractices or mislabeling of dried herbs. A survey of randomly selected dried herbs obtained from online marketplace and herbal powders obtained from traditional practitioners will be presented elsewhere in detail as this is beyond the scope of this work. Furthermore, we also checked the authenticity of the dried herbs and herbal powders included in this study using *matK* based DNA typing. It was found that of the 18 dried herbs tested, a total of 15 were identified as the same herb as it was mentioned on its label by online seller; however, plant species origin of one of the dried samples, which was sold to us in online marketplace with the spurious name 'Dashmool Bharad' (Sample—H7), was found to be that of *Tribulus sp.*. Similarly, the identity of two other herbal samples (Samples—H12 and H21 in Fig. 1) was found spurious (see footnotes in Table 2) either due to mislabeling or fraudulently replacement, indicating the incidences of malpractices in online herbal marketplace.

Furthermore, three of the five ayurvedic powders were also identified as that of the same herb’s origin as labeled by local ayurvedic practitioner; however, the identity of two of the samples was found spurious (Supplementary Fig. 2, Supplementary Table 4). These findings were confirmed by careful examination of high BLAST scores of the IDs of each specimen tested with multiple specimen vouchers entries in ncbi database, and also by inclusion of reference samples for some of the specimen tested in this study (Supplementary Fig. 3, Supplementary Table 5). These results provided initial DNA based evidence of the instances of malpractices in online herbal marketplace, these findings; however, need to be confirmed by comparing with a high fidelity reference database of authentic, dried medicinal herbs being used in various systems of plants based medicines around the world, which still needed to be done. Further investigation on such mislabeling or fraudulently replacement of dried herbs in online herbal marketplace will be presented elsewhere in detail as this is beyond the scope of this work. This study provides a novel, validated method of DNA isolation from dried aromatic herbs and herbal powders, which could be used as an economic alternative to successfully extract PCR quality DNA from such plant materials. The presented milk protocol was an “improved” protocol compared to the standard CTAB protocol of DNA isolation as shown in this study. Nevertheless we have not compared this protocol with alternative DNA extraction protocols and commercial kits; it would be interesting to see whether it outperforms even the alternative extraction protocols and available commercial kits for plant DNA extraction. In its current format, our method seems to be labor-intensive in terms of time relative to the commercial kits, but it is possible that inclusion of novel step of pretreatment of plant samples with skim milk may even enhance the success rates and efficiency of such kits in future.

This study further present a set of robust *matK* primers which could be used as plant barcoding resource in future studies. While validating the utility of our method of plant DNA isolation and typing in a brief molecular survey of randomly selected dried herbs obtained from online marketplace and herbal powders obtained from local ayurvedic practitioner, we also noticed the instances of malpractices or mislabeling of dried herbs and herbal powders, which are reported in this study and further seek an urgent attention. This also indicates the urgent need for a global effort to develop a high fidelity reference database of authentic, dried medicinal herbs which are being used in various systems of plants based medicines around the world.

**Data availability**

All sequencing data that support the findings of this study have been deposited in the National Center for Biotechnology Information “GenBank” (https://www.ncbi.nlm.nih.gov/) with the accession codes MN006706 to MN006768. All other data supporting the findings of this study are available within the paper and its supplementary information files.

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Author contributions
S.K.V. wrote the paper, conceived and designed the experiments, standardized the protocols and analysed the data. N.B. assisted S.K.V. in performing the experiments. S.K.V. conceived and led the project.

Competing interests
The authors declare no competing interests.

Additional information
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