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1 # Comprehensive analysis using DNA metabarcoding, PCR, and HPLC
2 # unveils the adulteration in Brahmi herbal products

3

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22

23 # Abstract

24 *Background:* The herbal products market is expanding and creating a bottleneck for raw
25 materials. Hence, economically motivated adulteration has a high prevalence. DNA barcoding
26 and species-specific PCR assays are now revolutionising the molecular identification of herbal
27 products and are included in a number of pharmacopoeias for the identification of raw
28 materials. High-throughput sequencing with barcoding advances toward metabarcoding, which
29 enables the identification of unintentionally or intentionally unlabelled plant material present
30 in herbal products. Brahmi is one of the most commercially significant and nootropic
31 botanicals, with great controversy over the terms "Brahmi" being used to describe both *Bacopa*
32 *monneri* (BM) and *Centella asiatica* (CA) species.

33 *Purpose:* This study evaluates DNA-based methods for Brahmi herbal products with the
34 traditional HPLC-based analytical approach in order to assess their effectiveness.

35 *Methods:* We employed a species-specific PCR assay, DNA metabarcoding using *rbcL*
36 minibarcode, and HPLC to detect the presence of the Brahmi (either BM or CA) in eighteen
37 market samples. All the methods have been validated using in-house blended formulations.

38 *Results:* Comprehensive analysis of all three methods revealed the presence of 22.2%, 55.6%,
39 and 50.0% of Brahmi by PCR assay, DNA metabarcoding, and HPLC, respectively, in Brahmi
40 market formulations, whereas blended formulations only exhibited targeted plant species with
41 all three methods.

42 *Conclusion:* Species-specific PCR can be used as a cost-effective and rapid method to detect
43 the presence of the Brahmi, while in high-throughput methods, DNA metabarcoding can be
44 used to detect the presence of widespread adulterated botanicals, and further, bioactive
45 compounds could be detected by HPLC. These results emphasise the need for quality control

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46 of the marketed Brahmi herbal products as well as the implementation of all methodologies in
47 accordance with fit for purpose**.**
48
49 *Key Words:* Adulteration, *Bacopa monneri,* Brahmi, *Centella asiatica,* DNA metabarcoding**,**
50 Species-specific PCR assay
51
52

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53 # Introduction

54 Traditional medical knowledge was gleaned and updated through centuries of empirical
55 testing. The rapid expansion of the traditional medicine market is a burning issue on a global
56 scale, as the quality of botanicals is being compromised either unintentionally by
57 substitute/adulteration due to a lack of taxonomic knowledge, different vernacular
58 nomenclature, and cryptic taxa morphology or intentionally by economically motivated
59 adulteration (Ichim, 2019; Raclariu et al., 2018a). The use of taxonomic and analytical
60 techniques for the authentication of medicinal plants are widely acknowledged around the
61 world, but the regulatory framework and guidelines for quality assessment may vary based on
62 whether they are used as herbal products or dietary supplements (Joshi et al., 2017; Liu et al.,
63 2018; Sahoo and Manchikanti, 2013). The resolution power of the analytical methods is greatly
64 influenced by environmental factors, differences in processing and storage conditions. In
65 addition to that, analytical methods cannot differentiate between an untargeted adulteration and
66 substitution, however, the ability to accurately identify the therapeutic component makes them
67 important for quality assurance and consumer safety (Raclariu et al., 2017). There is a lack of
68 centralized authentication systems that provide taxon identification with high-resolution
69 power. The predominance of species-specific PCR assays (Noh et al., 2021; Sharma and
70 Shrivastava, 2016) DNA barcoding(Bansal et al., 2018; Seethapathy et al., 2015; Thakur et al.,
71 2019; Vassou et al., 2016), and metabarcoding (Raclariu et al., 2017; Raclariu et al., 2017;
72 Raclariu et al., 2018b; Seethapathy et al., 2019) based molecular authentication of medicinal
73 plants has recently undergone a revolution, mostly because of DNA's accessibility, consistency,
74 and independence from tissue characteristics, age, harvesting techniques, and storage
75 conditions. Chinese Pharmacopoeia, United States Pharmacopeia, British Pharmacopoeia,
76 Japanese Pharmacopoeia, and Hong Kong Chinese Materia Medica are advocating DNA-based
77 authentication around the globe (Wu and Shaw, 2022). Species-specific PCR assays and DNA

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78 barcoding can provide identification of single or targeted plants at a lower cost, while high-
79 throughput sequencing-based metabarcoding can concurrently identify multiple taxon from the
80 mixture of DNA obtained from herbal products.

81 Brahmi is herbal therapeutics mentioned in traditional medicine for the treatment of
82 neurological and psychiatric disorders like loss of memory, cognitive deficits, and impaired
83 mental function (Shinomol et al., 2011). Brahmi is a classic illustration of controversial
84 drugs/sandigdha dravya as *Bacopa monnieri* L. (BM) (also known as waterhyssop, herb of
85 grace, Brahmi, thyme-leaved gratiola, Indian pennywort) and *Centella asiatica* L. (CA) (also
86 known as Mandukparni, Asiatic pennywort, Jalbrahmi, and Gotu kola), because of parallel
87 evolving knowledge systems, currently used polynomial nomenclature in Sanskrit, varying
88 perceptions in various communities, and vernacular equivalent (Keshari, 2021). The
89 therapeutic effectiveness of both is very similar despite having different family classifications
90 and taxonomy is due to having similar functional groups in secondary metabolites (Kashmira
91 J Gohil, 2020). Around the globe $320 million market value estimated for Brahmi by 2026.
92 The Asia-Pacific area is the largest trading sector for Brahmi, with India and China being major
93 exporters (https://www.industryarc.com/Research/Brahmi-Market-Research-507354).
94 Traditional markets, as well as Brahmi consumption, are prosperous in Northern America and
95 Europe due to natural thus fewer side effects ideology, traditional value, and accessible
96 availability (https://articlewire24.wordpress.com/2017/12/28/brahmi-market-size-and-
97 industry-forecast-2025-market-shares-and-strategies-of-key-players/).

98 In our previous studies, we have used plant DNA metabarcoding (Pandit et al., 2021)
99 and species-specific primers (Travadi et al., 2022) for the detection of adulteration in herbal
100 formulations. In this study, we investigated adulteration in Brahmi market samples using three
101 different approaches including species-specific PCR assay, metabarcoding and HPLC. The first
102 objective of this study was to develop a rapid, affordable and simple PCR-based assay for the

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103 detection of BM or CA in Brahmi herbal products. The second objective was to detect a wide
104 range of botanical adulterations in Brahmi herbal products using a DNA metabarcoding
105 approach using an in-house developed rbcL minibarcode. The third objective is to employ
106 established analytical techniques to confirm the presence of bioactive components. All of the
107 three techniques have been validated by mimicking possible blended formulations. The aim of
108 this study is to evaluate DNA-based methods for Brahmi herbal products by comparing them
109 with the conventional HPLC-based analytical strategy advocated by the Indian Pharmacopoeia.

110 Materials and Methods

111 Sample description

112 *Centella asiatica* (CA) and *Bacopa monnieri* (BM) plants were collected from the Maharaja
113 Sayajirao University (MSU), Vadodara, India with the help of taxonomists. Herbarium
114 vouchers of both plants were developed and submitted at the institutional herbarium with the
115 following voucher specimen IDs: CA: BG-181130-0005; BM: BG-181130-0018. Further, a
116 total of 18 market samples of different companies tagged as 'Brahmi churna/powder and vati'
117 were procured from the local market and e-commerce (Table S1).

118 Preparation of simulated plant mixtures and DNA extraction

119 BM and CA whole plant species were shed-dried, grinded into a fine powder and mixed in
120 proportions (w/w%) as follows. 1) BS1: 100% BM powder 2) BS2: 100% CA powder 3) BS3:
121 75% BM powder in 25% CA powder 4) BS4: 50% BM powder in 50% CA powder 5) BS5:
122 25% BM powder in 75% CA powder for preparing 100 mg of simulated blended formulations
123 for the validation of PCR assay, metabarcoding and HPLC. Qiagen-DNeasy plant power pro
124 kit (Qiagen, Germany) was used for extracting DNA from the BM and CA blended whole plant
125 materials as well as from the market samples. DNA was extracted as per the manufacturer's
126 instruction from 100 mg BM and CA blended materials and 100 mg powder of market samples.
127 During DNA extraction, proper precautions were taken to avoid cross-contamination. The *rbcL*

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128 gene was amplified to ensure the DNA quality using primers and thermal cycler conditions as
129 described by Travadi et al. (2022).

130 Development of PCR assay for detection of BM and CA

131 Designing of primers

132 For designing CA-specific primers, ITS sequences of BM and CA were retrieved from the
133 NCBI and aligned using ClustalW. Alignment was done to find out nucleotides that differed
134 between the two species. CA-specific primers were designed by considering polymorphic sites.
135 Two online software, Oligocalc (Kibbe, 2007) and Primer 3 version 4.0.0 (Rozen and
136 Skaletsky, 2000) were used to examine primer sequences for their optimal characteristics such
137 as length, and melting temperature compatibility, GC content, hairpin formation, and secondary
138 structure formation. The NCBI primer BLAST tool was used to examine the specificity and
139 selectivity of the primers. For determining BM, sequence characterized amplified region
140 (SCAR) primers designed by (Yadav et al., 2012) were used in this study.

141 Optimization of PCR assay

142 The annealing temperature for BM and CA primers were optimized in such a way that
143 provided higher sensitivity with optimum specificity. A total of 20 µL reaction mixture was
144 prepared comprising of 10 µL KAPA HiFi HotStart ReadyMix (Roche); 1 µL forward and
145 reverse primers (5 pmol each); 2 µL total genomic DNA (10-15 ng/µL); 2 µL bovine serum
146 albumin (BSA) (2 mg/L); nuclease-free water for the make-up final volume. A thermal cycler
147 was run with the following conditions to optimize annealing temperature; initial denaturation
148 at 95°C for 3 minutes, followed by 30 cycles of denaturation at 98 °C for 20 seconds, annealing
149 temperature from 64 to 72 °C (with an interval of 2 °C) for 15 seconds and extension at 72 °C
150 for 20 seconds, and final extension 72 °C for 20 seconds. For determining primer's sensitivity,
151 0.001, 0.01, 0.1, 0.25, 0.5, and 1 ng of genomic DNA of BM and CA were amplified with
152 optimized PCR conditions. To ensure the specificity and efficacy of optimized PCR assays,

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153 inter-species amplification using a mixer of BM and CA plant material was also executed. PCR
154 assays were performed with BM and CA-specific primers and optimized PCR conditions using
155 10-15 ng of DNA from prepared simulated blended formulations. Optimized PCR conditions
156 were further used for the detection of BM and CA in market samples.

157 **DNA metabarcoding**

158 For metabarcoding, we have designed *in-house* plant mini-barcodes targeting the *rbcL* gene
159 and it was validated by performing Next Generation Sequencing (NGS) assay on authenticated
160 plant mixture (data not shown here). The same primers were used in this study.

161 2.3.1 DNA metabarcoding of blended formulations and market samples

162 The optimized PCR reaction setup and thermal cycling condition were used as mentioned
163 above for metabarcoding of blended formulations and market samples. Amplified PCR
164 products were purified using AMPure XP beads (Beckman Coulter). Quantification of purified
165 PCR products was determined by Qubit 4.0 Fluorometer using the 1X dsDNA HS Assay Kit
166 (Thermo Fisher Scientific, MA, USA). Purified PCR products were mixed into equimolar
167 concentrations (100 pmol) and subjected to emulsion PCR (emPCR) for clonal amplification.
168 Emulsion PCR was carried out using Ion 520™ & Ion 530™ Kit-OT2 reagent solutions
169 (Thermo Fisher Scientific, MA, USA) with 400-bp chemistry as per manufacturer guideline.
170 Enrichment of template-positive Ion Sphere™ Particles (ISPs) was carried out using the Ion
171 OneTouch™ ES Instrument (Thermo Fisher Scientific, MA, USA). Sequencing was performed
172 on the Ion GeneStudio™ S5 Plus (Thermo Fisher Scientific, MA, USA) using the Ion S5™
173 sequencing solutions and reagents and loaded on the Ion 530™ sequencing chip (Thermo
174 Fisher Scientific). The efficacy of the metabarcoding assay was confirmed using simulated
175 plant mixtures prepared as mentioned above.

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176 **DNA metabarcoding data analysis**

177 The generated reads were filtered using PRINSEQ v0.20.4.31(Schmieder and Edwards,
178 2011) on the basis of average quality score and length. Reads with an average quality score Q
179 < 20 and reads with length <300 bp or >350 bp were discarded. Obtained filtered reads of each
180 sample were clustered using CD-HIT (Huang et al., 2010) at 99% identity. A representative
181 sequence of each cluster having a minimum of five reads was further analyzed using NCBI-
182 BLASTn (Altschul et al., 1990). To normalize data, the percentage of analysed reads is
183 considered as 100% for determination of percent distribution of each plant.

184 **HPLC analysis**

185 Authentic plant samples, simulated blended formulations and market samples were
186 extracted using methanol (1:10 w/v) to determine the presence of bacoside A (chemical marker
187 used to detect BM) and asiaticoside (chemical marker used to detect CA). Each extract was
188 filtered through a 0.22 µm pore size syringe filter (HiMedia Laboratories, Mumbai, India) and
189 20 µL of the filtered extract was injected into a high-performance liquid chromatographic
190 (HPLC). HPLC analysis was performed on the Shimadzu (Kyoto, Japan) system consisting of
191 an LC-20AP pump with SPD-M20A photodiode array detector (PAD) and C18 analytical
192 column (250 × 4.6 mm; i.d.: 5 µm). Analytical separations were carried out using a gradient of
193 acetonitrile (A) and water containing 0.05% (v/v) orthophosphoric acid (B) as the mobile
194 phase. The elution program was 0–25 min from 30:70 (A: B) to 40:60 (A: B), and 25–35 min
195 from 40:60 (A: B) to 60:40 (A: B). The flow rate was 1.5 mL/min and detection was performed
196 at 205 nm (Indian pharmacopoeia 2007, volume 3; Deepak et al. 2005). Commercially
197 available reference standard bacoside A and asiaticoside (Sigma-Aldrich) was injected (20 µL)
198 in HPLC in (1000 µg/mL) to obtain the chromatograms of both standards.

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199 # Results and Discussion

200 ## DNA extraction and rbcL gene amplification

201 The total DNA concentration ranges from 4.7–6.7 µg and 1.22-29.2 µg from the blended
202 mixture and market samples, respectively (Table S2). Herbal products are processed and
203 enriched with secondary metabolites such as polysaccharides, flavonoids, and polyphenols that
204 are reportedly co-precipitated along with the sheared DNA and inhibit PCR amplification
205 (Fazekas et al., 2009; Mishra et al., 2016a; Parveen et al., 2016). DNA integrity and quality
206 were checked using the *rbcL* gene from all the extracted DNA. In all the samples, we were
207 successfully able to amplify the *rbcL* gene, which revealed that the quality of extracted DNA
208 is suitable for PCR amplification (Fig. S1).

209 ## PCR based authentication of Brahmi market samples

210 The emergence and development of DNA-based technology has enabled the cost-effective,
211 rapid, and yet sensitive and species- specific PCR assays to authenticate medicinal plants.
212 Species-specific sequence-characterized amplified region (SCAR) markers, nuclear ribosomal
213 internal transcribed spacer (nrITS) sequences, and different plastid markers (e.g., rbcL, trnH-
214 psbA, and matK) have been extensively used to authenticate plant species(de Boer et al., 2015;
215 Mishra et al., 2016b; Sharma and Shrivastava, 2016; Xin et al., 2018). Earlier studies have
216 revealed a PCR-based assay for the authentication of herbal products. For instance, Noh et al.
217 (2021) developed a PCR assay for the authentication of medicinal mistletoe species; Zhang et
218 al. (2018) developed a PCR-based assay kit for the authentication of *Zaocys dhumnades* in
219 traditional Chinese medicine. Similarly, as mentioned earlier, we have also developed a PCR
220 assay for the detection of *Ocimum* sp. in Tulsi churna (Travadi et al. 2022).

221 To check the presence of either BM or CA in Brahmi products, we developed a species-
222 specific primer for CA using ITS region (sequence of primers not shown here), and for BM,
223 we used a SCAR-BM specific marker designed by Yadav et al. (2012). PCR assay optimization

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224 revealed that 65 °C is the optimum annealing temperature for both primer sets using KAPA
225 HiFi HotStart ready mix (Fig. S2). The results of the sensitivity experiment demonstrated that
226 the target DNA sequence was amplified successfully using BM and CA primers from 0.1 ng
227 of total DNA (Fig. S3). No cross-amplification was observed when BM and CA primers were
228 used with the blended formulation, and on the agarose gel, there was a band intensity gradient
229 proportional to the percentage of plant present in the blended formulations (Fig. 1a; 1b). This
230 established and validated the applicability of both primers. In addition, a PCR assay of Brahmi
231 market samples revealed that out of 18 samples, four samples (i.e. 22.2% samples) showed
232 amplification for the targeted species. Only two market samples with the labels 100 and 159
233 were found to detect CA, whereas two samples with the labels 220 and 223 had the presence
234 of BM (Fig. 2). The absence of the target bands in the rest of the market samples could be
235 attributed to the presence of counterfeit plant materials in substantial amounts.

236 **Metabarcoding of simulated blended formulations and market samples**

237 The raw data consists of a total of 138127 reads with a mean read length of 311 bp. A
238 total of 105353 (76.27%) reads passed our filtering quality criteria, and the mean read length
239 of filtered reads was 338 bp (Table 1). Table 1 shows the total raw reads and percentage of
240 reads acquired after filtering with the mean read length of each sample, as well as the
241 percentage of analysed reads. To mitigate the effect of sequencing errors that are known to
242 affect the Ion Torrent sequencing platform, we used a 99% OTU clustering threshold with a
243 minimum of five reads per cluster (Loman et al., 2012; Salipante et al., 2014). Substantiation
244 of the DNA metabarcoding method is required before its implementation for market samples.
245 Therefore, here we have performed metabarcoding of blended formations of BM and CA.
246 Blended formulations comprising 100% BM (BS1) showed 100% reads for BM, 100% CA
247 (BS2) showed 100% reads for CA, 75% BM +25% CA (BS3) showed 79.4% reads for BM and
248 20.6% reads for CA, 50% BM +50% CA (BS4) showed 48.4% reads for BM and 51.6% reads

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249 for CA, 25% BM + 75% CA (BS5) showed 23.9% reads for BM and 76.1% reads for CA (Fig.
250 3, 4). This data demonstrated the accuracy of our metabarcoding pipeline and its suitability for
251 market samples.

252 A total of 119 species, 113 genera and 41 families have been identified from the market
253 samples. Only four of the 18 market samples, labelled 100, 159, 223, and V47 showed 99.65%,
254 91.44%, 2.04% and 56.89% reads for CA, respectively, and five market samples labelled 102,
255 214, 215, 216, 219, 220, 223 showed 1.36%, 0.24%, 0.18%, 7.42%, 0.95%, 68.28% reads
256 respectively for BM (Fig. 3, Fig. 4). A total of 12 different plant species were shown to be a
257 prominent adulterant (covered >35% reads) in Brahmi market samples. For instance, sample
258 99 and 218 comprised 82.14% and 78.3% reads respectively for *Ipomoea batatas* (Family:
259 Convolvulaceae); sample 101 comprised 76.12% reads of *Eclipta alba* (Family: Asteraceae);
260 sample 102 comprised 61.6% reads of *Senna obtusifolia* (Family: Fabaceae); sample 203 and
261 223 comprise 79.3% and 34.6% reads respectively for *Momordica charantia* (Family:
262 Cucurbitaceae); sample 214 comprised 45.6% reads of *Ocimum tenuiflorum* (Family:
263 Lamiaceae); sample 215 comprised 41.36% reads of *Azadirachta indica* (Family: Meliaceae);
264 sample 216 comprised 51.95% reads of *Murraya koenigii* (Family: Rutaceae); sample 217
265 comprised 79.5% reads of *Xanthium sibiricum* (Family: Asteraceae); sample 219 comprised
266 65.23% reads of *Tephrosia candida* (Family: Fabaceae); sample 221 comprised 64.3% reads
267 of *Ziziphus spina-christi* (Family: Rhamnaceae); sample 222 comprised 94.38% reads of
268 *Trachyspermum ammi* (Family: Apiaceae); sample 224 comprised 84.7% reads of *Withania*
269 *somnifera* (Family: Solanaceae) (Fig. 3). Plant species and their percentage reads that are allied
270 with others in Fig. 3 are listed in Table S3. The number of species detected per sample ranged
271 from 2 to 28, with an average of 12.8 species in market samples.

272 A total of 44.4% samples exhibited 100% reads for non-targeted plants, 27.78%
273 samples showed 90 to 99.8% reads for non-targeted plants, and 55.6% samples showed reads

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274 in the range of 0.18 to 99.65% for Brahmi (either BM or CA or both) (Fig. 4a). At family level,
275 Fabaceae, Asteraceae, Lamiaceae, Solanaceae are prominent adulterants with presence in
276 minimum 10 samples with 77.8 %, 61.1%, 61.1% and 55.5% occurrences (Fig. 4b). On the
277 whole, this result emphasizes that a larger number of plant species have been detected in market
278 herbal products while we did not observe non-targeted plant species in-house prepared blended
279 formulations. The presence of unlabelled species might be deceiving, as DNA metabarcoding
280 is one of the highly sensitive methods that can detect even trace amounts of contamination. For
281 instance, pollen contamination because of wind-pollinated species during the stage of
282 cultivation and harvesting, during transport, storage, production, and packing other plant
283 species contaminated by inadequately cleaned containers, conveyors, and other equipment
284 (Newmaster et al., 2013; Ivanova et al., 2016; Liu et al., 2018). However, DNA metabarcoding
285 could only be applied for quality assessment to check the presence of labelled species and/or
286 other non-listed botanicals, substitutes, and fillers in herbal products. The final results are
287 influenced by a number of variables, including DNA quality and quantity proportional to the
288 plant species and its biasness; DNA that can be removed or degraded during processing; PCR
289 amplification bias due to poor primer fit and compatibility degree variation with different
290 species; library preparation; sequencing platform; metabarcoding data analysis parameters;
291 molecular identification algorithm; and reference databases (Pawluczyk et al., 2015; Staats et
292 al., 2016). NCBI GenBank databases comprise non-curated databases which might lead to
293 incorrect identification to the reference sequences at lower taxonomic level. However, NCBI
294 GenBank can be used for identification at higher taxonomic levels (Hinchliff and Smith, 2014).

295 **Detection of chemical markers of BM and CA in market samples**

296 A chemical marker-based authentication of BM and CA has been well demonstrated in the
297 Indian pharmacopoeia (Indian pharmacopoeia 2007, volume 3). In the present study, the most
298 commonly identified chemical markers bacoside A and asiaticoside were examined in Brahmi

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299 herbal products and blended formulations for validating DNA-based methods. Bacoside A is
300 composed of four different triglycosidic saponins: bacoside A3, bacopaside II, bacopasaponin
301 C, and the jujubogenin isomer, and it is the key bioactive constituent or chemical marker
302 responsible for the pharmacological effects of BM, while asiaticoside is the most abundant
303 triterpene glycoside found in the CA and is responsible for the pharmacological activities of
304 CA (Deepak et al., 2005; Shinomol et al., 2011).

305 In HPLC chromatogram, a reference standard bacoside A gave four major peaks
306 corresponding to bacoside A3 (1), bacopaside II (2), jujubogenin, isomer of bacopasaponin C
307 (3), and bacopasaponin C (4) between retention time 20 to 25 min and reference standard
308 asiaticoside gave a peak at retention time 5.08 min (Fig. S4). HPLC chromatogram of blended
309 formulations showed peaks in the asiaticoside and bacoside A region which corresponds to
310 their composition. For intense, 100% BM exhibited HPLC fingerprint at bacoside A region,
311 100% CA exhibited HPLC fingerprint at asiaticoside region, and admixture of BM and CA
312 exhibited HPLC fingerprints of both (Fig. S5). Samples 102, 214, 216, 219, 220, and 223
313 showed similar qualitative HPLC fingerprints in the bacoside A region demonstrating the
314 presence of BM. Samples 100, 159, and V47 showed a peak of asiaticoside obtained at a
315 retention time (Rt) of 5.08 min demonstrating the presence of CA. The remaining nine samples
316 (i.e. 50% samples) did not exhibit peaks corresponding to bacoside A or asiaticoside (Fig. 5).

317 3.1 Comparative results of all three approaches

318 Comparative results of PCR assay, DNA metabarcoding, and HPLC methods carried out in this
319 study for detection of BM and CA in herbal products are represented in Table 2. In sample 223,
320 BM and CA both are detected through metabarcoding, while by PCR-based approach and
321 HPLC only BM was detected. In samples 102, 214, 216, and 219, BM was detected by HPLC
322 and metabarcoding method but not through PCR-based approach and the presence of CA was
323 detected by HPLC and metabarcoding method but not through PCR based approach in sample

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324 V47. This might be due to the availability of lower copy numbers of SCAR (used for BM
325 detection)/ITS (used for CA detection) markers than *rbcL* markers, and in vati sample it could
326 be due to the presence of impurities in the extracted DNA, as we observed lower *rbcL* gene
327 amplification intensity in this sample (Fig. S1). Conclusively, presence of Brahmi (BM and
328 CA) was detected in four samples (22.22%) by PCR based assay, in ten samples (55.56%) by
329 metabarcoding and in nine samples (50.0%) by HPLC and in 8 samples (44.4%) BM and CA
330 was not detected by all three methods. There were labels on market products for *Bacopa*
331 *monnieri* on nine out of the 18 samples, but none had labels for *Centella asiatica* (Table S1).
332 However only four of them had BM, while one of them had CA instead of BM. From the
333 remaining 9 samples, where no species was mentioned on the label, BM and CA were found
334 in 3 and 2 samples, respectively. The low level of fidelity raises concerns about the safety and
335 reliability of Brahmi herbal products, especially in the context of discrepancies between
336 labelling and constituents.

337 Conclusion

338 Comprehensive analysis of species-specific PCR, DNA metabarcoding, and HPLC for
339 detection of BM and CA in herbal products revealed that DNA metabarcoding has benefits in
340 terms of detection of a wide range of non-targeted adulterations. While HPLC is able to detect
341 bioactive compounds, which are preferred in the identification and monitoring of the
342 therapeutic potential of drugs, a straightforward species-specific PCR approach, which is rapid,
343 affordable, and simple to analyse and handle, can overpower DNA metabarcoding and HPLC
344 in terms of cost and experimental simplicity. From the collection of raw materials to the final
345 furnished products, quality control of herbal products comprises various layers of investigation
346 and authentication. Species-specific PCR, DNA metabarcoding, and HPLC approaches have
347 distinct technological requirements, expenses, and levels of specialized knowledge required to
348 provide a quality assessment of herbal products with defined applicability and inapplicability.

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349 The implementation of multiple or orthogonal authentication approaches, which are aligned to
350 the concept of "fit-for-purpose," is the need of the hour in quality control and safety of herbal
351 products.

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358 Nitin Savaliya, Technical assistance from Thermo Fisher Scientific, for NGS instrument
359 handling and run setup.

360 Conflict of Interest

361 The sequence of CA primers and the PCR conditions have been applied for a patent
362 (Application Number-202221035088).

363 Author contribution

364 APS: Performed experiments, Data analysis, Writing and Editing manuscript, Validation of
365 final manuscript; TT: Performed molecular biology experiments, Data analysis, Writing
366 manuscript and validation; RP: Designed primers for metabarcoding, established
367 metabarcoding data analysis pipeline, Manuscript editing; SS: Designed primers for CA,
368 performed molecular biology experiments, Manuscript editing; CJ: Project administration,
369 Methodology, Supervision, and Review & Editing; MJ: Principal Investigator,
370 Conceptualization, Methodology, Supervision, and Review & Editing.

371 Data availability statement

372 All data generated or analysed during this study are included in this article and its
373 supplementary information files.

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485 # Captions for Figures

486 **Figure 1.** PCR assay of simulated blended formulations using *Bacopa monnieri* (BM) and
487 *Centella asiatica* (CA) specific primers. BM (100) =100% *Bacopa monnieri*; CA (100) = 100%
488 *Centella asiatica*; BM (75) & CA (25) = mixture of 75% of BM and 25% of CA; BM (50) &
489 CA (50) = mixture of 50% of BM and 50% of CA; BM (25) & CA (75) = mixture of 25% of

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490 BM and 75% of CA; N= No template control; M= 100 bp ladder. (a) PCR assay of simulated
491 blended formulations using BM primers (b) PCR assay of simulated blended formulations
492 using CA primers

493 **Figure 2.** PCR assay of Brahmi market samples using *Bacopa monnieri* (BM) and *Centella*
494 *asiatica* (CA) specific primers. 99, 100, 101, 102, 159, 203, 214 to 224, V47 = market sample
495 IDs; N= No template control; M= 100 bp ladder.

496 **Figure 3.** Relative abundance of botanicals at Genus level in Brahmi herbal products using
497 DNA metabarcoding.

498 **Figure 4.** *Bacopa monnieri, Centella asiatica,* and other non-targeted botanicals detected in
499 Brahmi herbal products (a) Heat map showing the relative abundance of targeted and nontarget
500 botanicals present within Brahmi herbal products (b) Family level distribution of botanicals
501 detected within Brahmi herbal products

502 **Figure 5.** HPLC chromatograms of Brahmi herbal products. Bacoside A components Bacoside
503 A3, Bacopaside II, Jujubogenin isomer of Bacopasaponine C, and Bacopasaponine C are
504 indicated by arrows labeled 1, 2, 3, and 4 respectively. The peak of asiaticoside is marked with
505 an arrow labeled with asiaticoside.

506

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507 **Table**

508 **Table 1.** Raw data of DNA metabarcoding

Samples	Before filtering: Total reads	Before filtering: Mean bp length	After filtering: Total reads	After filtering: % of total reads obtained	After filtering: Mean bp length	Total clusters	clusters having below 5 reads	clusters having > 5 reads	analysed reads (i.e., total reads having > 5 clusters	% of analysed reads
99	12204	288	7593	62.22	335	1013	207	806	6467	52.99
100	14039	308	9888	70.43	338	737	135	602	9065	64.57
101	21701	286	13478	62.11	336	1808	291	1517	11304	52.09
102	9484	307	6856	72.29	336	925	129	796	5774	60.88
159	726	315	592	81.54	339	143	19	124	432	59.50
203	2214	312	1788	80.76	339	500	50	450	1196	54.02
214	8115	319	7010	86.38	337	1246	139	1107	5503	67.81
215	5357	315	4555	85.03	336	604	45	559	3861	72.07
216	1461	313	1185	81.11	339	315	42	273	802	54.89
217	2568	315	2148	83.64	338	471	45	426	1585	61.72
218	3875	311	3142	81.08	336	532	77	455	2534	65.39
219	2166	319	1859	85.83	338	433	54	379	1372	63.34
220	3357	316	2810	83.71	338	530	74	456	2204	65.65
221	10615	322	9412	88.67	337	1076	156	920	8180	77.06
222	3448	315	2962	85.90	338	642	83	559	2208	64.04
223	3317	309	2720	82.00	340	866	96	770	1569	47.30
224	8854	323	7712	87.10	338	793	105	688	6774	76.51
V47	6589	263	3785	57.44	339	3147	1011	2136	2933	44.51
BS1	4673	317	4034	86.33	339	505	83	950	3470	74.26
BS2	2959	328	2720	91.92	339	233	35	422	2466	83.34
BS3	3386	318	2947	87.03	339	438	82	198	2449	72.33
BS4	2118	316	1810	85.46	339	305	43	356	1451	68.51
BS5	4901	323	4347	88.70	340	585	95	262	3656	74.60
Total	**138127**	**311.22**	**105353**	**76.27**	**337.96**	**17847**	**3096**	**15211**	**87255**	**63.17**

509 Where, BS1: 100% *Bacopa monnieri* (BM); BS2: 100% *Centella asiatica* (CA); BS3: BM (75%) + CA (25%);

510 BS4: BM (50%) + CA (50%); BS5: BM (25%) + CA (75%)

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511

512 **Table 2.** Comparative result of PCR assay, DNA metabarcoding and HPLC

	PCR assay using BM and CA primers		Metabarcoding		HPLC	
Sample	*Bacopa monnieri*	*Centella asiatica*	*Bacopa monnieri*	*Centella asiatica*	Bacoside A (*Bacopa monnieri*)	Asiaticoside (*Centella asiatica*)
99	-	-		-	-	-
100	-	+	-	**+ (99.65% read)**	-	+
101	-	-	-	-	-	-
102	-	-	**+ (1.36% reads)**	-	+	-
159	-	+	-	**+ (91.44% reads)**	-	+
203	-	-	-	-	-	-
214	-	-	**+ (0.24% reads)**	-	+	-
215	-	-	**+ (0.18% reads)**	-	-	-
216	-	-	**+ (7.42% read)**	-	+	-
217	-	-	-	-	-	-
218	-	-	-	-	-	-
219	-	-	**+ (0.95% read)**	-	+	-
220	+	-	**+ (68.27% read)**	-	+	-
221	-	-	-	-	-	-
222	-	-	-	-	-	-
223	+	-	**+ (12.24% read)**	**+ (2.04 % read)**	+	-
224	-	-	-	-	-	-
V47	-	-	-	**+(56.89% read)**	-	+
Absolute (relative) number of samples in which BM and CA was detected	4.0 (22.22%)		10.0 (55.56%)		9.0 (50.0%)	

513

514 + sign represents detection of respective plant species; - sign represents absence of respective plant species, %

515 reads of BM and CA obtained in DNA metabarcoding is also represented.

516

517

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Figures

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Figure 1

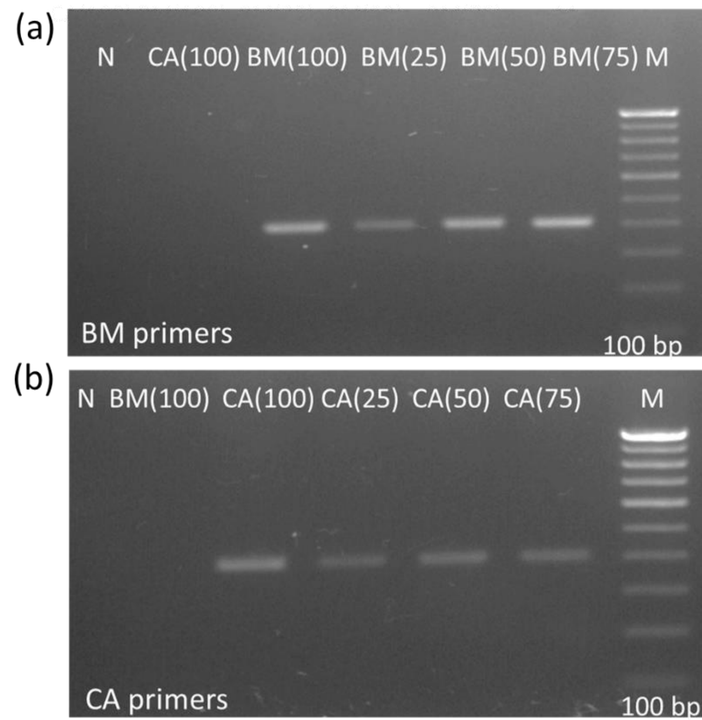

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Figure 2

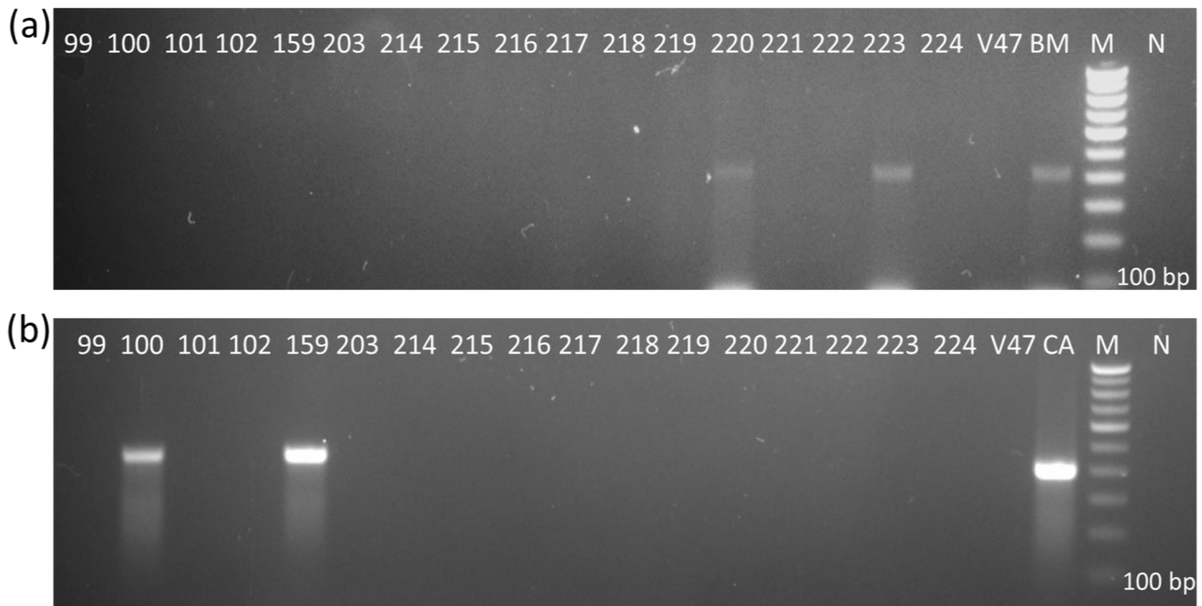

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Figure 3

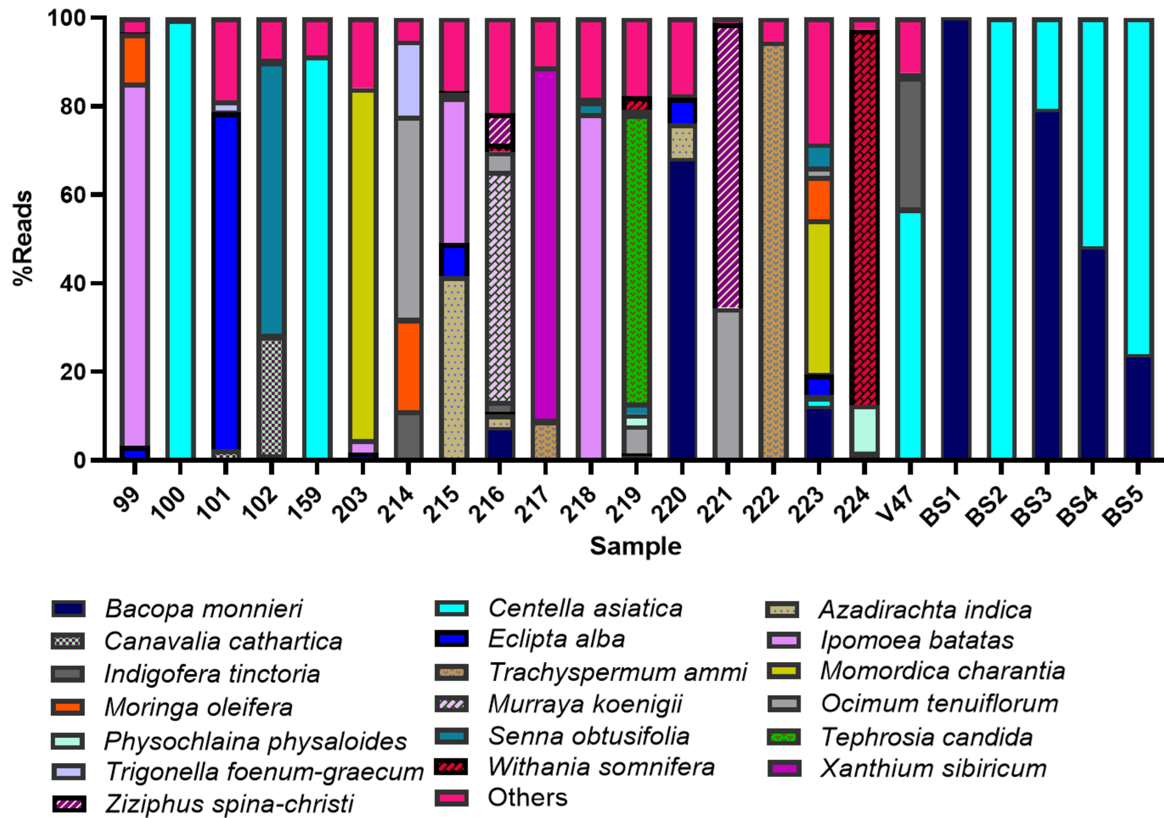

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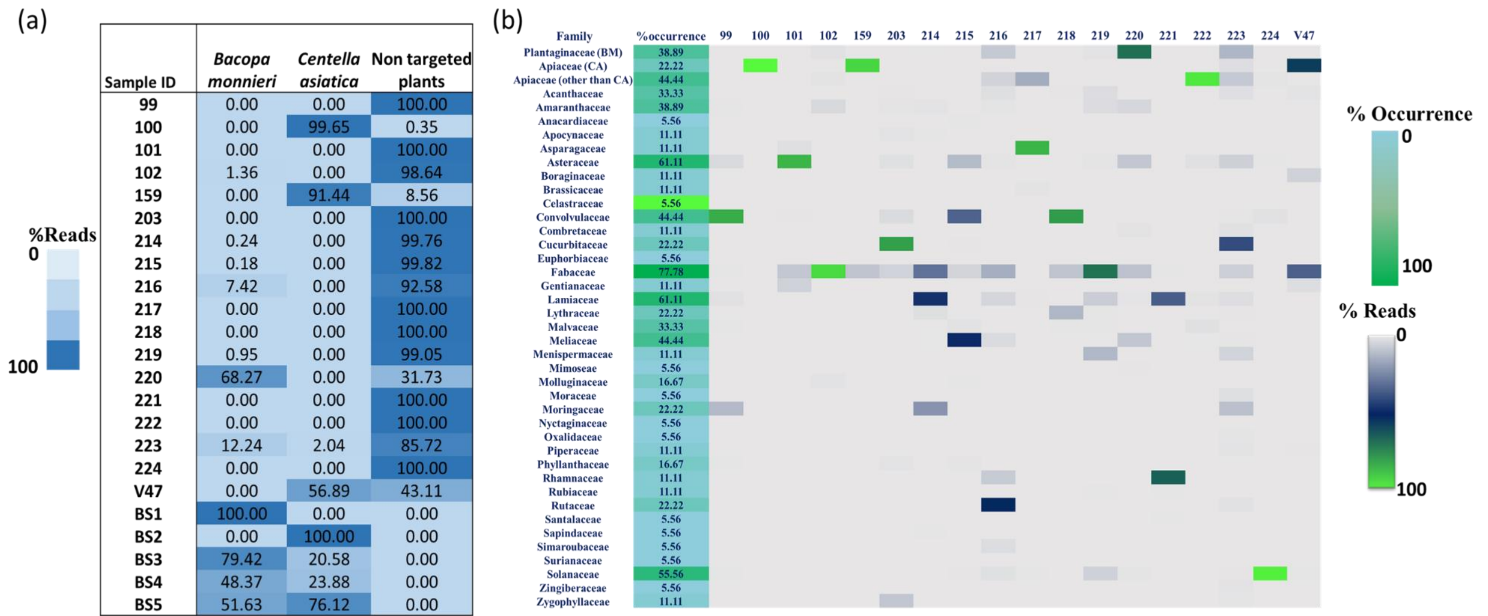
Figure 4


(a)

Sample ID	*Bacopa monnieri*	*Centella asiatica*	Non targeted plants
99	0.00	0.00	100.00
100	0.00	99.65	0.35
101	0.00	0.00	100.00
102	1.36	0.00	98.64
159	0.00	91.44	8.56
203	0.00	0.00	100.00
214	0.24	0.00	99.76
215	0.18	0.00	99.82
216	7.42	0.00	92.58
217	0.00	0.00	100.00
218	0.00	0.00	100.00
219	0.95	0.00	99.05
220	68.27	0.00	31.73
221	0.00	0.00	100.00
222	0.00	0.00	100.00
223	12.24	2.04	85.72
224	0.00	0.00	100.00
V47	0.00	56.89	43.11
BS1	100.00	0.00	0.00
BS2	0.00	100.00	0.00
BS3	79.42	20.58	0.00
BS4	48.37	23.88	0.00
BS5	51.63	76.12	0.00

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Figure 5