

AN EFFICIENT PROTOCOL FOR DNA EXTRACTION FROM FRESH AND HERBARIUM SPECIMENS OF HELIOTROPES.

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Abstract

High quality DNA is essential in various techniques of molecular systematic. Herbarium specimens are the valuable source of genetic information in this respect especially for those taxa which grow in areas which are difficult to access, very rare or endangered. However, the DNA obtained from the herbarium specimen is often highly fragmented or present in very low quantity. Most of the *Heliotropium* L. species produce large number of secondary metabolites. For isolating high quality DNA, free from secondary metabolites, a low cost but efficient protocol for different species of *Heliotropium* L. is developed. The DNA isolated with this protocol was successfully used for PCR based downstream applications such as gene amplification, DNA sequencing and Bar coding.

Keywords: Herbarium, secondary metabolites, extracted DNA, gene amplification.

Introduction:

Herbarium specimens are used to study various morphological investigations as they contain lot of information. They are also extensively used to carry out a variety of molecular studies particularly of those taxa whose fresh specimens are not easily available, either growing in areas which are difficult to access or being rare/ endangered. The Extraction of DNA is a very intricate and complex process especially the DNA obtained from herbarium specimen are highly fragmented and sometimes present in very low quantity. Fumigation (Metzger & Byers 1999), chemical preservatives, high temperatures or use of microwave oven for drying and pest control. (Hall 1981, Bacci *et al.* 1983) cause severe damage to DNA molecule. Air drying is considered to be better than the preservation of tissues in Silica gel or anhydrous CaSO₄. If the specimens are air dried up to 42°C (Taylor and Swann 1994) they contain an ample amount of high yielding DNA. The extraction of DNA and its subsequent use in molecular studies may also be problematic because of the presence of Polysaccharides, Phenols and other organic compounds (Porebski *et al.* 1997). A number of protocols have been established (Saghai Maroof *et al.* 1984; Doyle & Doyle 1990; Scott & Playford 1996; Haymes 1996; Porebski *et al.* 1997; Li *et al.* 2002; Sharma *et al.* 2000; Drabkova *et al.* 2002; Shepherd *et al.* 2002; Mogg & Bond 2003; Cheng *et al.*, 2003) to extract high quality DNA free of chemicals, impurities and contamination. The chemotypic heterogeneity among species may not permit total DNA yields from a single isolation protocol; even closely related species of the same genus may require different isolation protocols (Weishing *et al.*, 1995).

Heliotropium is a genus of about 270-276 species, cosmopolitan in distribution, represented in Pakistan by 23 species distributed in 11 sections (Nasir 1989). A variety of secondary metabolites have been isolated from the bioactive extract of *Heliotropium angiospermum* (Gilda *et al.*, 2009). Commercially available kits like

DNeasy plant mini kit (Qiagen Valencia CA, USA) give good results but are very expensive (Ahmed *et al.* 2009). The use of multiple extraction protocols is laborious, time consuming and expensive. All these problems necessitate the development of a protocol for isolating high quality DNA which is easy, rapid, inexpensive and less laborious. Here in the present study we described an easy, simple and cost effective CTAB based method that yields high quality genomic DNA from fresh as well as dry leaves of *Heliotropium*. This protocol can be applied to all the plants fresh or dried.

Materials and methods

Reagents and solutions

- Suspension buffer PH8 [0.1mM EDTA, 50mM Tris HCl, 100 mg PVP (Polyvinylpyrrolidone), 0.2% mercaptoethanol (freshly added just before use).]
- CTAB extraction buffer (2× CTAB: 50 ml of 1M tris-HCl, 150 ml of 5M NaCl, 50 ml of 0.25M EDTA, 10 g of CTAB and Distilled water to make up volume up to 500ml by maintaining Ph 8
- Chloroform: isoamyl alcohol (24:1)
- Isopropanol
- Tris-EDTA
- RNase, 20 mg/mL
- Sodium acetate 3M
- Ethanol 70% and 96%

DNA Extraction Protocol

1. Grind 0.2-0.3gm dried leaves to fine powder in ice cold condition in presence of 100mg PVP and 300 µL suspension buffer by using pre-chilled mortar and pestle (at-80°C).
2. Transfer the content in 2 ml micro centrifuge tubes and suspend in 300 µL of suspension buffer_for 30 min. to 2hours at 4°C.
3. Add 750 µL of preheated CTAB extraction buffer (64°C) to the contents and incubate with gentle agitation at 60°C for 1 hour.

4. Let the solution to cool down to room temperature and add 1 volume chloroform+ isoamyle alcohol (24:1)
5. Shake 5-10 min. at room temperature and Centrifuge at 13000 rpm for 10 min.
6. Carefully transfer the upper aqueous phase to 1.5ml new tube.
7. Repeat steps 4-6.
8. Add double volume of chilled isopropanol and keep at -20°C for 1 hour or overnight to precipitate the DNA. (longer the chilled treatment higher will be the precipitation)
9. Centrifuge at 13000 rpm for 10 min. at 4°C.
10. Discard the supernatants and wash the pellet with 70% chilled ethanol (500 µL)
11. Centrifuge at 12000 rpm for 15 min.
12. Discard the Supernatant and air dry the pellet.
13. Resuspend the pellet in 50-100 µL of TE. Add 5 µL of 20 mg/mL RNase. mix well and incubate for 30 min at 37°C.
14. Add 50µL of 3M Sodium acetate and 500µL ice-cold 96% ethanol and store at -20°C for 1 hour
15. Centrifuge in micro centrifuge at 13000 rpm for 13 min.at 4°C
16. Again, wash the pellet with 70% ethanol (200 µL) and air dry.
17. Suspend the pellet in 50-100 µL of TE buffer and store at - 20°C.

*Note: Use sterilized sand when grinding hard leaves or leaves with thick trichomes such as *H.ophioglossum* and *H.dasy carpum*.

PCR Amplification and Analysis of Extracted DNA

PCR reactions were performed in a thermo-cycler (Meigene I, Germany) in a 30 µL volume containing 0.2 µL of 1U of Taq polymerase, 2.25 µL of 1.5mM MgCl₂, 1.2 µL of 0.4mM DNTPs, 2 µL of 50ng of DNA, 1.8 µL of each primer (10 µM), 3 µL of 1x plant buffer and 17.75µL deionized water. The PCR profile conducted for amplification of rbcL, matK, trnL-trnF, psbA-trnH and trnS-trnG was as follow: pre-denaturation at 95°C for 3 min. followed by cycles of denaturation at 95°C for 20 s, annealing (variable according to primer) 35s, and extensions at 72 °C for 35 sec. with final extension at 72°C for 5 min.(Table 1).

The extracted DNA samples were run on 1% agarose gel stained with 4ul Visulana (Molequole-on New Zealand)/Ethidium bromide in horizontal gel electrophoresis assembly (Cleaver Scientific HU10, UK). The samples were compared with DNA size marker (Gene Direx, Taiwan) of 1Kb. The gel was documented using Gel Documentation System (UVI Tech, UK) and gel picture was recorded. DNA was quantified by measuring optical density at A260 and A280 with a Nanodrop Spectrophotometer (Implene, Germany).

Table 1. Primers used to Amplify cpDNA.

Name of Gene/intergenic spacer	Primer	Product size
rbcL	F- CTGATATCTTGGCAGCATTC R-CGCCCTTCATTACGAGCTT	1204bp
matK-U-IRKIM matK-U-3FKIM	F-ACCCAGTCCATCTGGAAATCTTGGTTC R- CGTACAGTACTTTTTGTGTTTACGAG	800bp
psbA-trnH	F- C GCGCATGGTGGATTACAAATCC R- GTTATGCATGAACGTAATGCTC	500bp
trnL-trnF	F-CGAAATCGGTAGACGCTACG R- ATTTGAACTGGTGACACGAG	850bp
trnS-trnG	F-GCCGCTTTAGTCCACTCAGC R-GAACGAATCACACTTTTACCAC	650bp

Results and Discussion

The concentration of DNA obtained from herbarium as well as fresh specimens ranged from 43-598 ng/ µL, which is sufficient to amplify the different regions of Chloroplast genome including more than 50 years old herbarium specimen (Table 3). The highest concentration of DNA was obtained in fresh specimen of *H.ophioglossum* whereas *H.europaeum* showed the high concentration and ratio in

herbarium specimen. However, the DNA extracted from herbarium specimen by CTAB method (Doyle & Doyle 1990) showed low yield (Table 2) and failed to form amplicon.

As far as the DNA extraction is concerned, *Heliotropium* is a problematic genus containing a large number of secondary metabolites (Gilda *et al* 2009). Even the extracted DNA from fresh sample of *Heliotropium ophioglossum* was failed to amplify the required genome

when it was subjected to PCR amplification. Therefore, the current protocol is good enough to amplify PCR fragments up to 1kb (Figs. 2-6). The age of specimen is not of much importance and does not affect the concentration or ratio of extracted DNA. However, method of preservation of plant specimen including drying procedure and the age of leaves at the time of preservation has the major effect on the yield of DNA. All the herbarium specimens used in this study were air dried using corrugated sheets/ simple drier having electric bulbs of 40/60 watt. The DNA extracted from herbarium specimens utilizing different protocols (Doyle & Doyle 1990; Scott & Playford 1996; Haymes 1996; Porebski *et al.*, 1997) was low yield, fragmented, coloured or highly viscous. The coloured DNA indicated the contamination of phenolic compounds adhered to DNA, while high viscosity was due to the presence polysaccharides which directly or indirectly interfere with the enzymatic reactions (Weishing *et al.*, 1995). DNA extraction from herbarium material is very delicate and intricate process. The first and the most important step in the protocol is grinding or crushing of plant material, instead of using liquid nitrogen we used pre chilled mortar and pestle (at -80°C) for crushing and grinding dried leaves along with the suspension buffer which homogenized the

tissue sufficiently and further suspension at 4°C for 30min. to 2hours gave good results, in case of hard leaves or leaves with thick trichomes sterilized sand was used during grinding. However, many protocols use liquid nitrogen for homogenization of plant tissue or Pulverizing plant material with a mixer mill (Csaikl *et al.*, 1998) our current protocol is therefore, low cost as we did not use liquid nitrogen. For the removal of secondary metabolites high concentration of PVP was used and 2% merceptoethanol was freshly added. Washing two times or more (when required) with chloroform: isoamyle alcohol (24:1) was also very effective to remove contaminations and proved amenable to PCR amplification. Moreover, chilled treatment for longer period (overnight) gave high precipitation of DNA. Another problem which we encountered during DNA isolation from herbarium specimens was the rapid degradation. The DNA extracted from herbarium material degraded more rapidly than isolated DNA from fresh tissue and its effectiveness decreases very rapidly with time as also reported by Jansen *et al.*, (1999). Therefore, we used standard TE (1M Tris-HCl and 0.5M EDTA) for DNA storage at -20°C or at -30°C (for longer period). It is recommended to use the isolated DNA (from herbarium) within a year.

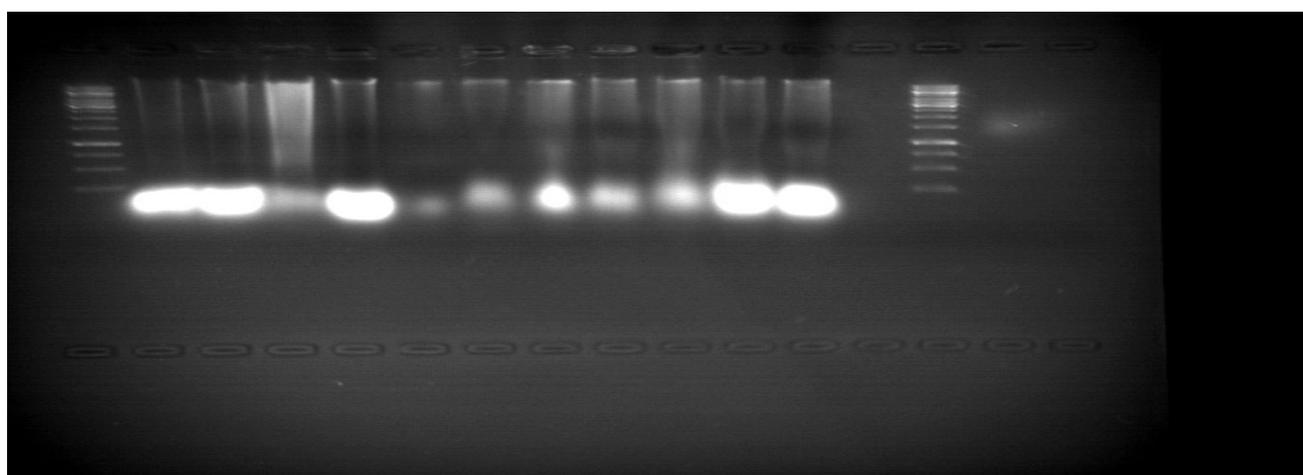


Fig. 1. Gel picture of extracted DNA [Lane: 2-3 *H.marifolium*, 4,5 *H.cbulicum*, 6-8 *H.europeum*, 9,10 *H.calcareum* 11,12 *H.rariflorum*. Lane: 1 and 14 DNA markers

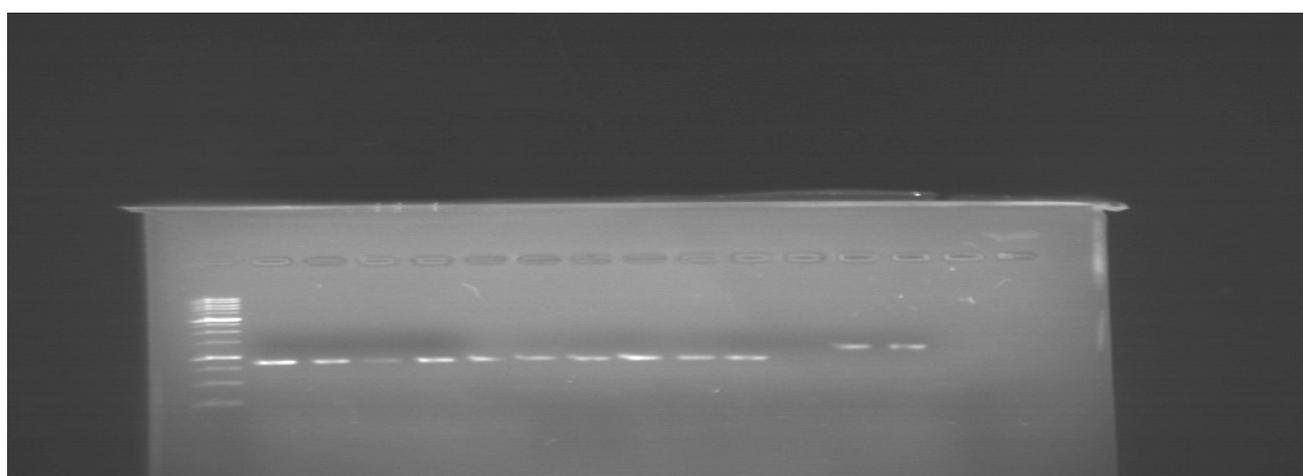


Fig. 2. Amplification of rbcL Gene: Lane: 2-14 *H.marifolium*, *H.cabulicum*, *H.europeum*, *H.calcareum*, *H.subulatum*, *H.ophioglossum* with their replicates. Lane:1 DNA marker

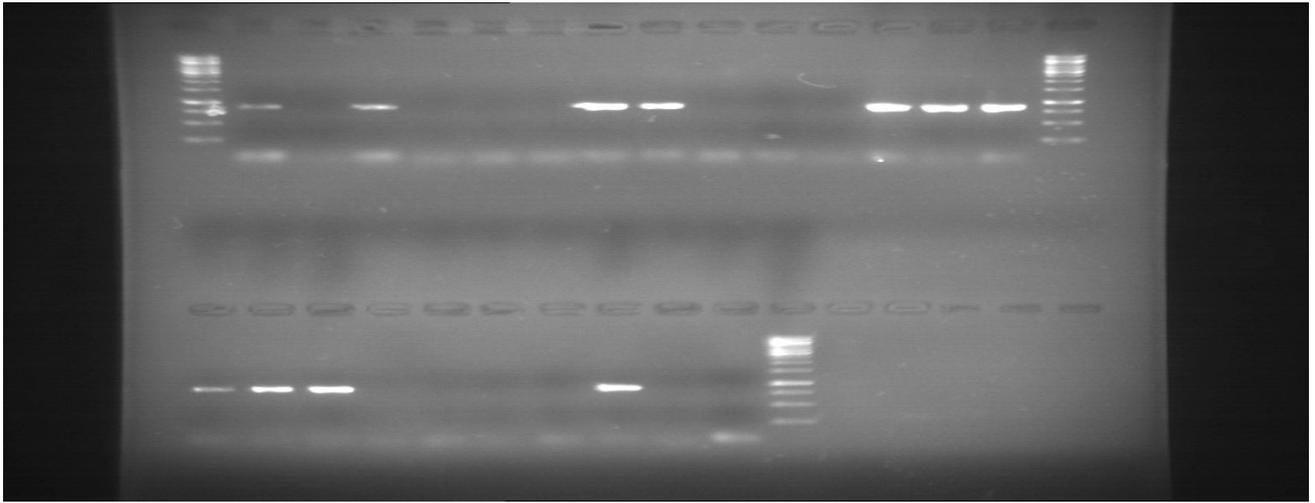


Fig. 3. Amplification of matK Gene Lane: 2-14 (upper row) 1-10 (lower row) *H.marifolium*, *H.cabulicum*, *H.europeaum*, *H.calcareaum*, *H.subulatum*, *H.ophioglossum* with their replicates. Lane: 1, 15 (upper row) 11 (lower row) DNA markers

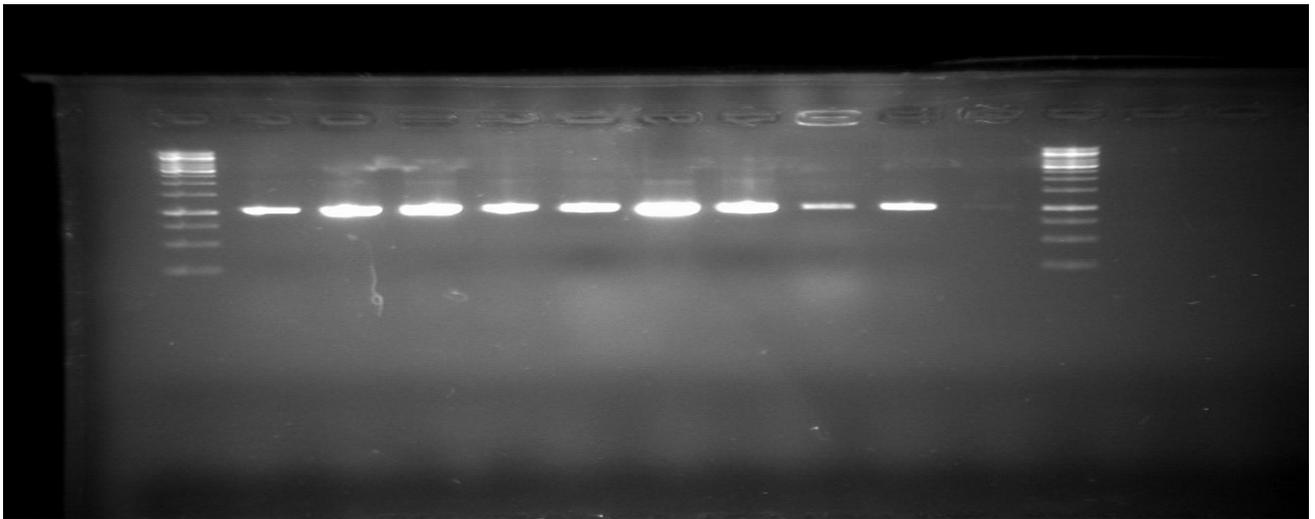


Fig. 4. Amplification of trnL-trnF intergenic spacer Lane: 2-12. *H.marifolium*, *H.cabulicum*, *H.europeaum*, *H.calcareaum*, *H.rariflorum*, *H.ophioglossum* with their replicates. Lane: 1,13: DNA markers

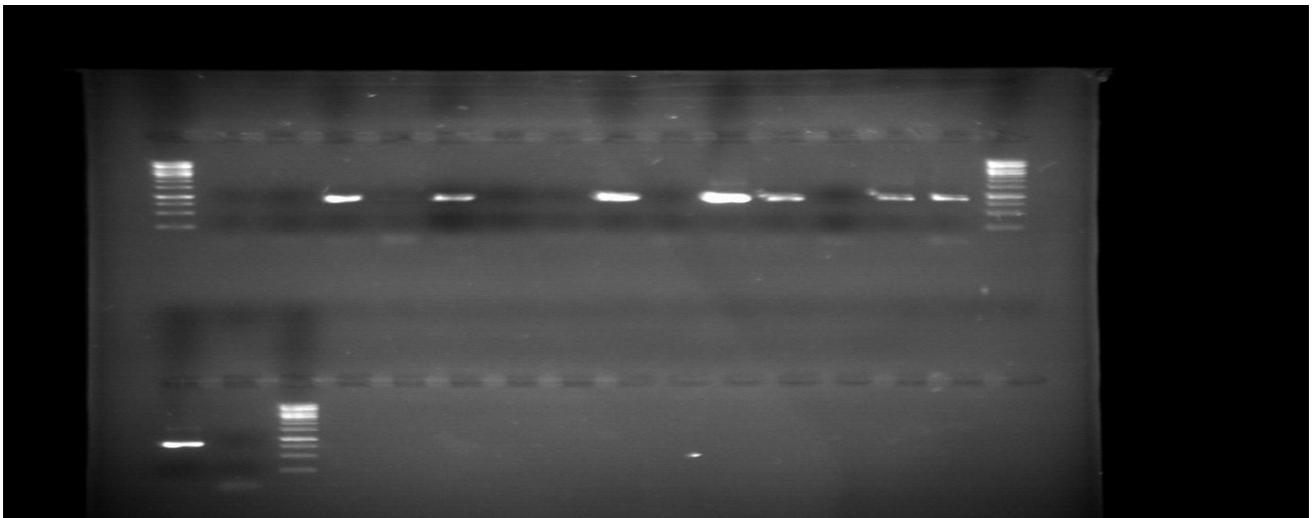


Fig. 5. Amplification of trnS-trnG intergenic spacer Lane: 3,5,8,10,11,13 and 14 (upper row) Lane:1 (lower row) *H.ophioglossum*, *H.cabulicum*, *H.calcareaum*, *H.marifolium*, *H.subulatum*, *H.europaeum*, *H.rariflorum*. Lane: 1,13 (upper row) Lane: 3 (lower row) DNA markers

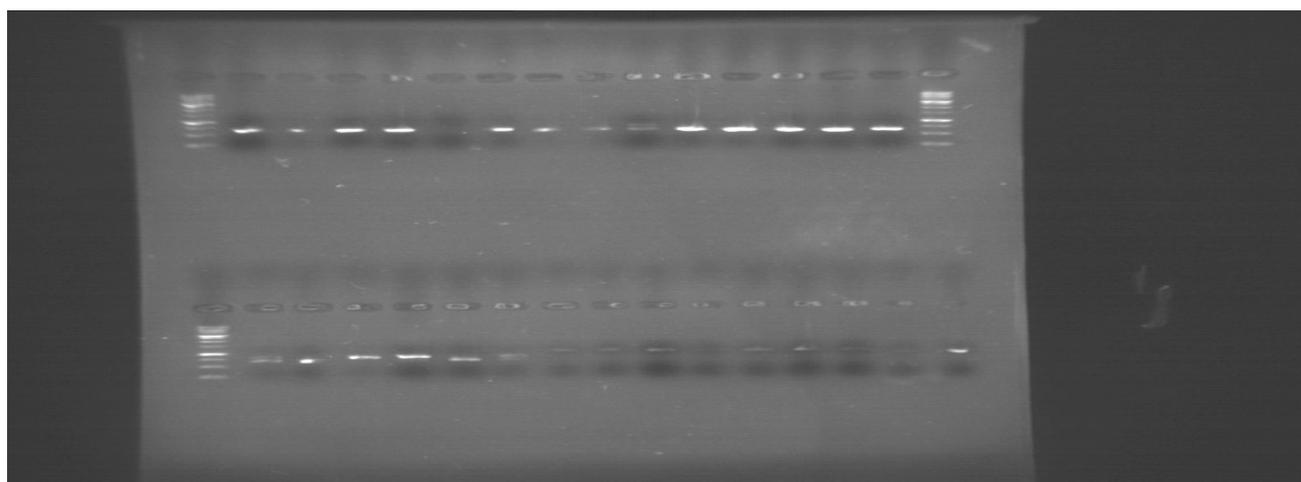


Fig. 6. Amplification of psbA-trnH intergenic spacer Lane: 2-5,7-14 (upper row) Lane:2-7 (lower row) *H.ophioglossum*, *H.cabulicum*, *H.calcareum*, *H.marifolium*, *H.subulatum*, *H.europaeum*, *H.rariflorum* with their replicates Lane: 1,15 (upper row) Lane: 1 (lower row) DNA markers

Table 2. A comparison between CTAB protocol and current protocol.

Taxon Name	Collection Date	DNA conc.(ng/μL)	DNA conc.(ng/μL)	Ratio (A260/A280)	Ratio (A260/A280)
		CTAB protocol	current protocol	CTAB protocol	Current protocol
<i>H.cabulicum</i>	1976	61	85.5	1.12	1.62
<i>H.cabulicum</i>	1974	25.5	43	0.8	1.65
<i>H.calcareum</i>	1970	86	93	1.2	1.53
<i>H.calcareum</i>	1983	73	91	0.88	1.56
<i>H.europaeum</i>	1968	22	152	1.5	1.69
<i>H.europaeum</i>	1974	96	56	1.133	1.63
<i>H.europaeum</i>	1970	53	67	1.21	1.5
<i>H.ophioglossum</i>	1986	42	95	1.45	1.53
<i>H.ophioglossum</i> (Fresh)	2014	177	598	1.7	1.77
<i>H.marifolium</i>	1987	88	143	1.1	1.65
<i>H. marifolium</i>	1983	91	122	0.92	1.58
<i>H.rariflorum</i>	1987	35	103	1.23	1.8
<i>H.rariflorum</i>	1959	37	113	1.4	1.76
<i>H.subulatum</i>	1985	67	102	1.22	1.63
<i>H.subulatum</i> (Fresh)	2014	155	140	1.68	1.76

Note: Two fresh specimens of *H.ophioglossum* & *H.subulatum* were used as control.

Table 3. Voucher information for specimens used in this study.

Taxon Name	Collector and Number	Collection Date	Collection Locality	Herbarium
<i>Heliotropium cabulicum</i>	Nasir & Siddiqui 17610	1976	Sadda Kurram	KU
<i>Heliotropium cabulicum</i>	Qaiser & Sultan ul Abedin 17719	1974	Mansahra	KU
<i>Heliotropium calcareum</i>	Farooqui & Qaiser 17653	1970	Hyderabad	KU
<i>Heliotropium calcareum</i>	Saood umer& Nazimuddin 18236	1983	Chilas	KU
<i>Heliotropium europaeum</i>	Sultan ul Abedin 17834	1968	Darasanachano	KU
<i>Heliotropium europaeum</i>	Sultan ul Abedin & Qaiser 17857	1974	Kasur	KU
<i>Heliotropium europaeum</i>	Qaiser & Ghafoor 17836	1970	Peshawar	KU
<i>Heliotropium ophioglossum</i>	Razia Ahmed 18227	1986	KU campus	KU
<i>Heliotropium ophioglossum</i>	Samina Naurin 88028	2014	Kaneez Fatima society	KU
<i>Heliotropium marifolium</i>	Qaiser& Tahir Ali 18227	1987	Mirpurkhas	KU
<i>Heliotropium marifolium</i>	Qaiser & Nazimuddin 18986	1983	Bhawalpur	KU
<i>Heliotropium rariflorum</i>	Khatoon & Ayesha 16674	1987	KU campus	KU
<i>Heliotropium rariflorum</i>	Jafri 18068	1959	Malir	KU
<i>Heliotropium subulatum</i>	S.I.Ali 18174	1985	Malir	KU
<i>Heliotropium subulatum</i>	Samina Naurin 88229	2014	Gulistan e johar	KU

Conclusion

The current method of DNA extraction is easy, quick, inexpensive and less laborious which is easily applicable not only on the specimens of *Heliotropium* but other plants also. Moreover, fresh specimens give better yield of quality DNA (Table 2) with this protocol.

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