IJPSR (2019), Volume 10, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 12 October 2018; received in revised form, 21 June 2019; accepted, 26 June 2019; published 01 July 2019

MICROWAVE ASSISTED EXTRACTION OF BIOLOGICAL COMPONENTS OF *CROCUS SATIVUS* WITH DNA BARCODING TECHNOLOGY

Tina Sharma [*1] and Mukesh Kumar Thakar [2]

Department of Forensic Science and Toxicology [1], Department of Forensic Science, Chandigarh University, Gharuan - 140413, Punjab, India.
Department of Forensic Science [2], Punjabi University, Patiala - 147002, Punjab, India.

Keywords:

DNA Barcoding,
Saffron adulteration,
Forensic Science, Herbal medicine

Correspondence to Author:
Tina Sharma

Assistant Professor,
Department of Forensic Science and Toxicology, Department of Forensic Science, Chandigarh University,
Gharuan - 140413, Punjab, India.

E-mail: sharmatina1989@gmail.com

ABSTRACT: Saffron is derived from the flower of *Crocus sativus* as a dried stigma. Kashmiri saffron is the most expensive herb and in sky-scraping demand by herbal and pharmaceutical industries due to which the illegal trade is prevailing in the industry to earn instant Profit. Detection of adulteration and comparison of genuine saffron *vs.* fake saffron is the crucial aspect which needs a scientific solution. Samples were taken Mogra (A), Lacha (B), Iranian (C) and unknown grade of saffron (D) from Pampore and Anantnag region of Kashmir. The DNA from the samples was extracted with the help NucleoSpin® Plant II Kit and were amplified with the help of reported universal primers of matk, rbcL and MatK, and bidirectional Sanger sequencing was performed. Only ITS gene showed positive extraction and amplification results. ITS gene was capable of distinguishing the interspecific as well as intraspecific variations. The blind trial conducted on unknown grade sample revealed the presence of substituent, which turned out to be Safflower, *i.e. Carthamus tinctorius* as observed through nucleotide blast search. The site-specific variations were observed, and the nucleotide sequences of ITS obtained during the study were submitted to the NCBI gene bank with accession number MH984846, MH984847, and MH984848. The examination of saffron with DNA Barcoding methodologies may contribute to health care communities a new validated tool to authenticate the samples and improve the efficacy of herbal medicine used to treat various ailments.

INTRODUCTION: Saffron is the world's most expensive spice and is derived from the dried stigma of *Crocus sativus*. The Crocus genus belongs to family Iridaceae of order Asparagales and comprises of about 70 to 100 species [2]. It is the main distributers are Mediterranean Europe and Western Asia.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(7).3482-86

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(7).3482-86

Iran accounts for about 90% of the world assembly of saffron. In India, Kashmir (J & K) is the largest producer of saffron, which is mainly grown in Pampore and Anantnag. The grades of saffron can be categorized into two major grades, *i.e.* 'Mongra' and "Laccha."

Mogra saffron contains stigma alone while Laccha saffron contains stigmas attached with parts of the style [3]. The expensive nature of saffron is related to its production as 1 kilogram of saffron requires 110,000-170,000 flowers. The herbal plants with economic importance are often smuggled from one state to another or from one border to another to earn instant profit [3].

The illegal activities also include adulteration or substitution of expensive genuine herb with a cheaper similar looking herb which violates quality assurance and can be dangerous in consumption as well [4].

Saffron contains more than 150 volatile and many nonvolatile active components which are responsible for its medicinal properties and its higher demand in the herbal industry. The volatile components are terpenes, terpene alcohols, and their esters among which safranal ($C_{10}H_{14}O$) is the main component. Non-volatile compounds comprise crocins $C_{44}H_{64}O_{24}$, crocetin $C_{20}H_{24}O_4$, picrocrocin $C_{16}H_{26}O_7$, and flavonoids. Crocin, glucosyl esters of crocetin are water-soluble carotenoids which are meant for saffron's characteristic color. Picrocrocin, a glycoside of safranal, is responsible for the bitter taste of the spice and Safranal, is responsible for its characteristic aroma. Saffron's taste and hay-like fragrance are due to picro-crosin and safranal. Its golden yellow-orange color is the result of crosin which is trans-crocetin di-ester, and its IUPAC name is 8,8-diapo-8,8-carotenoic acid [5]. Saffron is of different quality and strength. The difference lies in the amount of style present and age of saffron. More style present in saffron means the saffron is less strong. Saffron is categorized into various grades.

Grades of Iranian Saffron:

1. Sargol- strongest grade, stigma only
2. Pushal- low grade, style present with the stigma
3. Bunch- lowest grade, a large amount of style present with red stigma.

Grades of Kashmiri Saffron:

1. Mogra saffron- strongest among all the saffron worldwide, Stigma alone
2. Laccha saffron- Low-grade Kashmiri saffron, stigma attached with style

Kashmiri saffron is very expensive, and its demand is increasing day by day due to which it is very prone to adulteration [3]. It is sold in the range of Rs.300-500 per gram in Kashmir. According to a study on saffron sold in Kashmir, only 52% is genuine, 30% is low grade and 17% adulterated. Adulteration also includes selling of mixes of different saffron grades. High-grade Kashmiri saffron is mostly mixed with low-grade cheaper Iranian saffron and is then sold as pure Kashmiri saffron.

DNA barcoding is one such technology which utilizes short sequences ranging from 300 to 1000 bp sequences to identify the biological specimens which aim and orients its applications in conservation biology, phylogenetic, ecology, taxonomic studies, Pharmaceutical studies as well as forensic studies to identify species in illegal trade [5]. DNA barcoding is well established in animals as well as plants species. DNA barcoding could be utilized to identify and delineate the species in question [6, 7]. The present study is carried out to identify adulterant or any substituent among samples of saffron with DNA Barcoding Methodology.

MATERIALS AND METHODS: Dried commercial samples of *C. sativus* marketed as whole stigmas and from different geographic proveniences from Pampore, Anantnag and Srinagar district of Kashmir valley were also purchased and to conduct the blind trial the samples were also purchased from local herbal vendor of Chandigarh herbal market. Different varieties of samples claimed to be Mogra saffron, Kashmiri saffron, Iranian Saffron, and local sample (Chandigarh) were marked as Sample A to D.

Samples Collected:

- Sample A: (Mogra Saffron)
- Sample B: Lacha Saffron)
- Sample C: Iranian saffron.
- Sample D: (Unknown grade)

DNA Barcoding: 1 g of saffron stigmas were extracted by immersing in 5 mL of absolute ethanol at ambient temperature overnight, in the dark, followed by Microwave-assisted extraction. The extracts were sealed in 2 mL vials and stored at 4 ºC. The genomic DNA from extracts was carried out with the help of NucleoSpin® Plant II Kit (Macherey Nagel). Plant DNA Kit was used for DNA extraction according to the manufactured protocol.

Quality of the extracted DNA was determined using gel electrophoresis PCR amplification reactions with PCR thermal cycler (GeneAmp PCR System 9700 with 1x 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers were carried out in a 20 µl reaction volume. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequences analysis was carried out using Bioedit and Mega 7.0 editing software for by assembling both forward and reverse sequences for each gene amplified. The homology of sequences obtained during experimentation is confirmed by using NCBI blast tool. The Insilco analysis among crocus genus was carried out between obtained ITS sequences and Sequences obtained through NCBI for C*rocus etruscus, Crocus biflorus, Crocus cancellatus, Crocus laevigatus.*

RESULTS: The DNA was successfully extracted from samples types A, B, C and was further amplified for three markers matk, rbcL and ITS. The DNA extraction of dry samples are little cumbersome but all the samples were successfully extracted. The samples were further amplified with polymerase chain technology for three specific gene markers, *i.e.* matk, rbcL, and ITS.

All the samples failed to show amplification for matk and rbcL except for ITS marker, which showed successful amplification in all the samples. The amplified DNA was sequenced, and sequences obtained were edited and assembled with the help of Mega 7.0 software. The sequences showed nucleotide composition, as shown in **Table 1**.

TABLE 1: SHOWING NUCLEOTIDE COMPOSITION OF CROCUS GENUS

	T(U)	C	A	G	Total
FIMPT-CS1-ITS	20.4	31.1	19.0	29.5	643.0
FIMPT-CS2-ITS	20.4	31.1	19.0	29.5	643.0
FIMPT-CS3-ITS	20.4	31.1	19.0	29.5	643.0
Crocus etruscus	20.2	29.3	20.2	30.3	634.0
Crocus biflorus	18.5	30.9	19.5	31.0	686.0
Crocus cancellatus	19.2	30.1	19.5	31.3	652.0
Crocus laevigatus	19.2	30.1	19.5	31.3	652.0
Avg.	19.7	30.5	19.4	30.4	650.4

The sequences obtained during the present study were further submitted to NCBI and accession numbers were obtained as MH984846, MH984847, and MH984848. The sequences obtained were analyzed using blast n of NCBI to compare the sequences with an existing database. The taijama neutrality test showed 595 segregating sites with 2.6 taijama test statistics. The barcoding gap was found to be much higher in case of ITS genes as the interspecific genetic distance was almost null as compared to interspecific genetic distance was calculated with P- distance to be 0.5537 as shown in **Table 2** and **Fig. 1** and **Fig. 4**.

TABLE 2: SHOWING TAIJAMA NEUTRALITY TEST AND GENETIC DISTANCE IN CROCUS GENUS

Number of sequences	Segregating sites	Nucleotide Diversity	Taijama test statistics	Interspecific Distance ITS	Intraspecific Distance
7	595	.5537	2.624352	0.5537	0.0

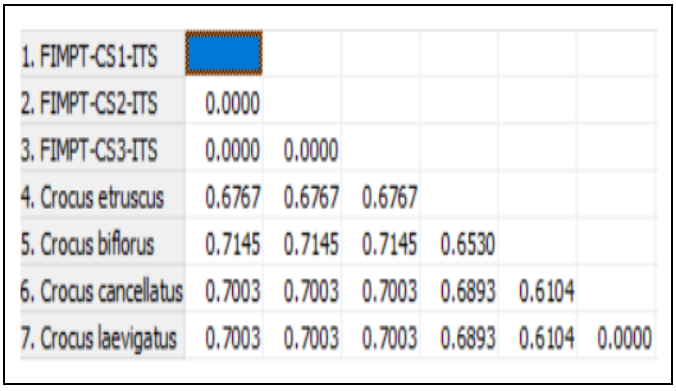

FIG. 1: SHOWING PAIR WISE INTERSPECIFIC DISTANCE BETWEEN *CROCUS SATIVUS* AND RELATED SPECIES WITH ITS BARCODING MARKER

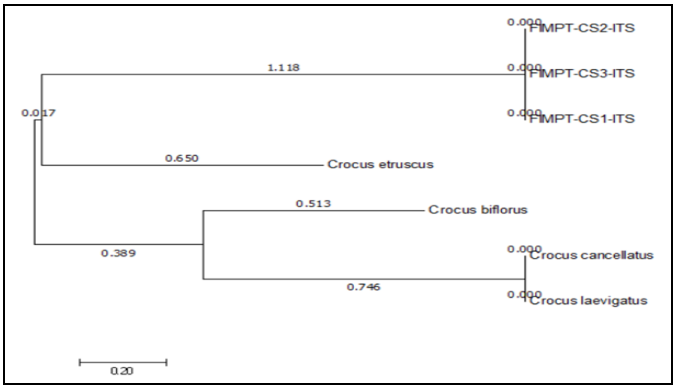

FIG. 2: SHOWING PHYLOGENETIC RELATIONSHIP WITH MAXIMUM LIKELIHOOD TREE USING KIMURA 2 PARAMETER WITH *CROCUS SATIVUS* AND RELATED SPECIES WITH ITS MARKER

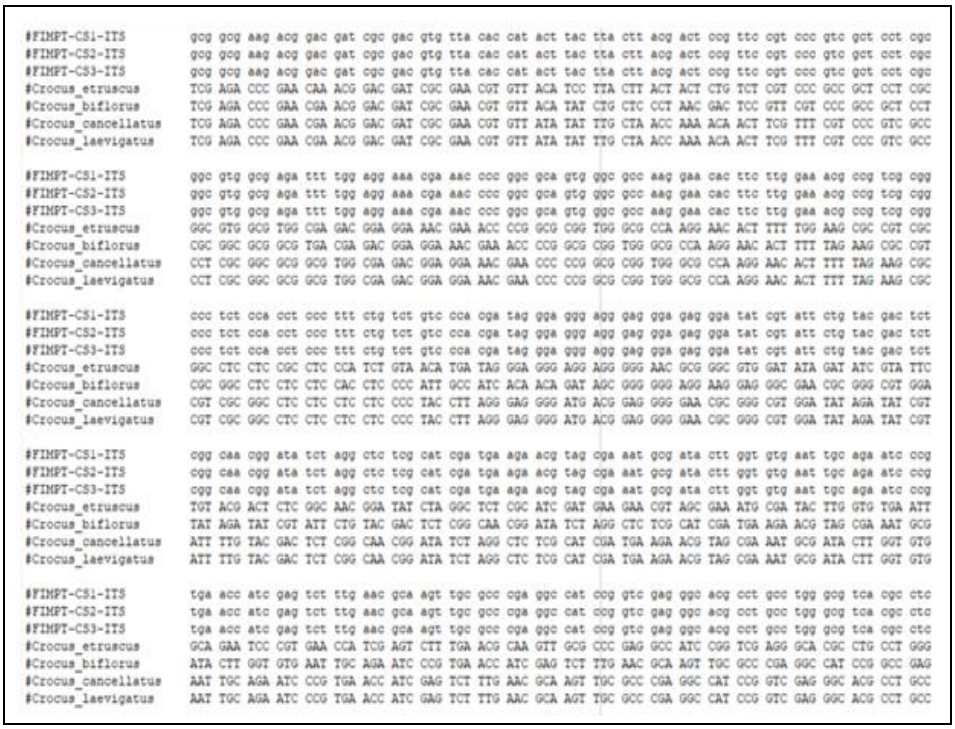

FIG. 4: MULTIPLE SEQUENCE ALIGNMENT OF CROCUS GENUS WITH THE HELP OF MEGA 7 SOFTWARE

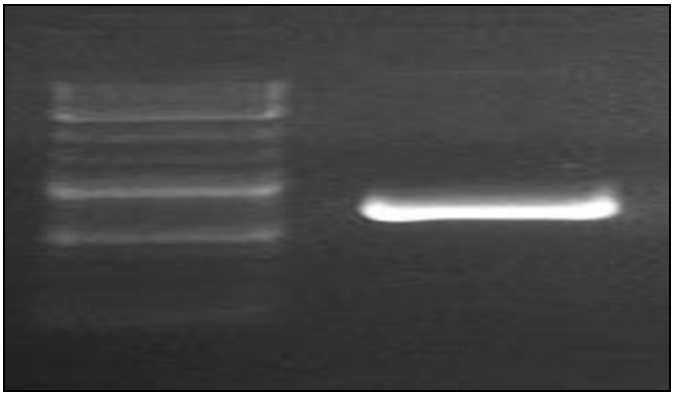

FIG. 3: SHOWING EXACTION AND AMPLIFICATION OF GENE IT'S IN 3kb LADDER

All the sequences obtained from sample A, B, C showed 100% homology with ITS, but sample D surprisingly showed 99.2% similarity with *Carthamus tinctorius, i.e.* Safflower. It was then observed that the *Carthamus tinctorius, i.e.* Safflower colored similar to the saffron plant is being sold on herbal outlets of Chandigarh with prize quite similar to the genuine herb.

Phylogenetic Tree: The phylogenetic relationships in the *Crocus sativus* with interspecific species of the same genus (*Crocus etruscus, Crocus biflorus, Crocus cancellatus, Crocus laevigatus*) was established by the combination of *ITS* **Fig. 2**, with the high average supporting values for nodes each tree showed a "fan" shape, with closely related species clustering together, whereas distantly related species relatively scattering. The analysis of the relative distribution of distances by K2P pairs using Taxon DNA showed that each loci have a global barcode gap. ITS gene marker shows the potential to distinguish the closely related species of crocus genus with the help of maximum likelihood phylogenetic tree method.

CONCLUSION: The present study was conducted to compare and evaluate the quality of saffron samples. Only ITS sample was successfully amplified and sequenced to produce DNA barcodes of the ITS gene. The gene was also tested for its resolution capability to distinguish the closely related species with the help of maximum like a hood phylogenetic tree. The study showed the successful identification of adulteration and substitution of the herbal product with similar looking cheaper *Carthamus tinctorius*. The preliminary examination of saffron with DNA Barcoding methodologies may contribute to the health care communities with validated PCR based methodology to authenticate the herbal samples and improve the efficacy of herbal

ACKNOWLEDGEMENT: The authors are thankful to UGC for providing fellowship to carry out the present research work.

CONFLICT OF INTEREST: Nil

REFERENCES:

1. Bakshi H, Sam S, Rozati R, Sultan P, Islam T, Rathore B, Lone Z, Sharma M, Triphati J and Saxena RC: DNA Fragmentation and Cell Cycle Arrest: A Hallmark of Apoptosis Induced by Crocin from Kashmiri Saffron in a Human Pancreatic Cancer Cell Line. Asian Pac J Cancer Prev 2010; 11(3): 675-9.
2. Dhar AK, Sapru R and Rekha K: Studies on saffron in Kashmir: Variation in Natural Population and its Cytological Behavior. Crop Improvement (India) 1988; 12-33.
3. Nehvi FA, Wani SA, Dar SA, Makhdoomi MI, Allie BA and Mir ZA: Biological interventions for enhancing saffron productivity in Kashmir. Acta Horticulturae 2007; 739: 25-26.
4. Thakar MK, Sharma T and Sahajpal V: Illegal trade of Medicinal Plants approaching wildlife forensics: Indian Scenario. Editorial Board 2018; 130.
5. Hebert PD, Penton EH, Burns JM, Janzen DH and Hallwachs W: Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *A. fulgerator*. Proc of Nat Acad of Sci 2004; 41: 14812-17.
6. Group CP, Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, van der Bank M, Chase MW, Cowan RS, Erickson DL and Fazekas AJ: A DNA barcode for land plants. Proceedings of the National Academy of Sciences 2009; 31: 12794-7.
7. Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X and Luo K: Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PloS one 2010; (1): e8613.

How to cite this article:
Sharma T and Thakar MK: Microwave assisted extraction of biological components of *Crocus sativus* with DNA barcoding technology. Int J Pharm Sci & Res 2019; 10(7): 3482-86. doi: 10.13040/IJPSR.0975-8232.10(7).3482-86.