Advances in Bioresearch
Adv. Biores., Vol 13 (5) September 2022: 39-44

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.13.5.3944

Advances in Bioresearch

ORIGINAL ARTICLE

Antioxidant, antimicrobial activity, characterization of phytochemicals, and phylogenetic analysis using barcoding regions ITS and matK – in *Seetzenia* lanata (Willd.) Bullock

Piyush Panwar[1], Manisha Mathur[2], GP Singh[3]
[1]Department of Botany, University of Rajasthan, Jaipur, Rajasthan.
[2]Department of Botany, GD Girls College, Alwar, Rajasthan.
[3]Department of Botany, University of Rajasthan, Jaipur, Rajasthan.
Email: piyushpanwar243@gmail.com

ABSTRACT
The Zygophyllaceae family is known for many important medicament plants. Till now 285 numbers of species and 22 genera has been reported as a member of Zygophyllaceae. Among them, 10 to 11 numbers of species are reported in the Thar Desert, India. In India Seetzenia lanata (Willd.) Bullock is reported as a rare plant species of Zygophyllaceae. Plants were classified in many different ways. Among them, DNA barcoding is one of the best ways to phylogenetically classify all plants specie. Study till now reports a scarcity of study on antimicrobial, antioxidant activity, Secondary metabolite, and phylogenetic analysis. In the present study phylogenetic analysis through Matk and ITS was analyzed with their closely related species. Along with that, DPPH antioxidant assay, antimicrobial activity through isopropyl alcohol extract, and a Preliminary study on secondary metabolite were analyzed. Resulting in Satisfying antimicrobial activity against P. chrysogeum, T. rubrum, and M. canis, alkaloid was identified by iodoplatinate reagent. matK gene of Seetzenia lanata (Willd.) is showing a close relationship with Tribulus terrestris and Balanites maughamii and in ITS it is on a separate clade.
Keywords- *Zygophyllaceae, DNA barcoding, Internal transcribed spacer, ITS, MaturaseK, matK, DPPH, antimicrobial, Alkaloid.*

Received 10.07.2022 Revised 26.07.2021 Accepted 21.08.2022

How to cite this article:
P Panwar, M Mathur, GP Singh. Antioxidant, antimicrobial activity, characterization of phytochemicals, and phylogenetic analysis using barcoding regions ITS and matK – in *Seetzenia* lanata (Willd.) Bullock.. Adv. Biores. Vol 13 [5] September 2022. 39-44

INTRODUCTION

The Zygophyllaceae family is known as a family of flowering plants. Some of their plants have the potential to grow dominantly in arid and semiarid environments. It is reported that 285 numbers of species and 22 genera are present in Zygophyllaceae [1]. Among them, 10 to 11 numbers of species are reported in the Thar Desert, India. Approximately 85% of the Thar desert is distributed in Gujarat and Rajasthan, India [2-3]. Plants are playing an important role in the survival and flourishment of human civilization. They are a good source of nutrition like simple to complex carbohydrates, fatty acids, protein antioxidants, secondary metabolites, etc. Plants also play an important role in the treatment of many diseases. In India *Seetzenia lanata* (Willd.) Bullock is reported as a rare plant species of Zygophyllaceae [2]. *Seetzenia lanata* (Willd.) is distributed between Jaisalmer and Jodhpur districts of Rajasthan, and Kutch, Gujarat, India [5-6].

Classification of plants is done in many different ways among them phytochemical classification and morphological classification were dominantly accepted by researchers. This type of classification has some limitations. The presence of similar morphological features and the impact of environmental factors on plants may provide false results and misidentification. Nowadays phylogenetic analysis of plants through DNA barcodes is getting attention [3]. It is known for its accurate results and acceptance by researchers. DNA barcodes reasons are short comparatively more conserved sequences used in the establishment of phylogenetic relationships. In the present study MaturaseK gene (matK) and Internal

transcribed spacer (ITS) are used to establish phylogenetic relationship with other plant species of Zygophyllaceae [8-9].

Antioxidants are simple or complex compounds that are capable of inhibiting oxidation reactions. An oxidation reaction can cause mutation that may lead to cancer, apoptosis, senescence, etc [10-11]. There are many ways to identify antioxidant activity in plants. The study of nonenzymatic antioxidant activity with DPPH antioxidant assay is a useful, easy and effective way. In the present study different concentrations of plant, extract is used to study antioxidant activity in *Seetzenia* lanata (Willd.) Bullock [4]. Resistance against antibiotics is increasing day by day. It is essential to search for less toxic and effective antibiotics. Plants are a good source of antimicrobial compounds. Crude extract isolated from polar solvent like isopropyl alcohol can help in the study of antimicrobial activity present in *Seetzenia* lanata (Willd.). Presence of secondary metabolites like alkaloid in *Seetzenia* lanata (Willd.) Bullock is not fully known. In the present study, de-fatted phytochemicals were isolated and analyzed from Seetzenia lanata (Willd.). Thin layer chromatography separated compounds were identified by reagents like iodoplatinate, DPPH, 50% Sulphuric acid, etc.

MATERIAL AND METHODS

Chemicals and material

Ammonia (Molychem, India), sulfuric acid (Molychem, India), n-hexane (Molychem, India), chloroform (Molychem, India), ethanol (Changshu Hongsheng Fine Chemicals, China), acetone (Molychem, India), diethylamine (RFCL Ltd., India), potato dextrose agar (Sisco Research Laboratories Pvt. Ltd., India), nutrient agar (Sisco Research Laboratories Pvt. Ltd. India), platinic chloride hexahydrate (SRL, India), DPPH (HiMedia Laboratories Pvt. Ltd. India), DMSO (Molychem, India), TLC silica gel 60 F254 (Sigma, Germany), DNA isolation kit (Bio Bee stores Pvt. Ltd., India.), UV-visible spectrophotometer (Lab Junction, India), Quartz Cuvette (ML-Apex Labs, India), Thermal Cycler (Applied Biosystems Veriti, USA), ABI 3730xl sequencer (Applied Biosystems, USA), Qubit 3.0 (Thermo Fisher Scientific, USA).

Collection and identification of plant material

Seetzenia lanata (Willd.) Bullock plant was ingathered from Bagga, Phalodi, District Jodhpur, Rajasthan, India (Latitude: 27.143876 and Longitude: 72.328292). Identification of the plant was done by the Botanical Survey of India (***BSI) Jodhpur, Rajasthan, India***. Collected plants were exhaustively washed under the tap and distilled water. After washing and shade drying, the plant samples were powdered for phytochemical studies like DPPH assay, antimicrobial activity, and thin layer chromatography. For barcoding and phylogenetic analysis, a fresh plant sample was used for isolation, purification, and amplification of DNA.

FASTA format barcode sequence of other closely related species from the family of Zygophyllaceae was downloaded from the NCBI database. Species and accession number detail are - *Fagonia laevis* (MF963564.1 matK, AY641634.1 ITS), *Fagonia arabica* (KM276890.1 matK, AY641618.1 ITS), *Tribulus terrestris* (MH211038.1 matk, MT322923.1 ITS), *Balanites maughamii* (JF270650.1 matK, MH990661.1 ITS).

Isolation and amplification of DNA

Isolation of DNA was done by a DNA isolation kit developed by Bogar Bio Bee stores Pvt. Ltd., India.

Homogenization- Contamination free and fresh plant sample (50 mg) was mixed and crushed with lysis buffer (500 µl). Ribonucleases (4 µl) and neutralization buffer (500 µl) was added to crushed plant sample and incubated in a waterbath for 30 minutes at 65˚C. The incubated mixture was then centrifuged for 10 minutes at 10,000 rpm. After that without disturbing the pellet, the supernatant was transferred into a fresh centrifuge tube. Chloroform isoamyl alcohol (600 µl) was added with supernatant (600 µl) and mixed vigorously. Followed by that centrifugation was done for 10 minutes at 10,000 rpm and the aqueous phase was collected in a fresh centrifuge tube.

Binding- The aqueous phase (600 µl) was then mixed thoroughly with kit-based binding buffer (600 µl) and incubated for 5 minutes at room temperature. Contents were then transferred to a spin column and placed in a collection tube (2 ml). After that centrifugation was done for 2 minutes at 10,000 rpm and flow-through was abandoned.

Washing- To wash contamination two different types of washing buffers were used separately (500 µL of washing buffer I and washing buffer II). Followed by centrifugation for 2 mins at 10,000 rpm, and flow through was discarded.

Elution- Consequently, the dried spin column was transferred into a new microcentrifuge tube. After that elution buffer (100 µl) was added to the middle of the column and incubated for two minutes at room

temperature. Consequently, the column was centrifuged for 2 minutes at 10,000 rpm. Now buffer along with DNA in the microcentrifuge tube was measured with the help of Qubit 3.0.

Polymerase Chain Reaction (PCR)- For amplification of specific DNA sequence PCR or polymerase chain reaction was used (Thermal Cycler from Applied Biosystems™ Veriti™, USA). reaction solution Constructed with forward primer and reverse primer (Table 2), deionized water, Taq DNA polymerase, dNTPs, MgCl2, and bromophenol blue were mixed with isolated DNA. Depending on the length of primers and presence of nucleotides, the temperature involved in PCR cycles was set (Table 1).

Sequencing and data analysis

Sequencing was done by ABI 3730xl sequencer from Applied Biosystems, USA (after removing unincorporated PCR primers and dNTPs). For submission of gene sequence GenBank at National Center for Biotechnology Information or NCBI (NCBI, http://www.ncbi. nlm.nih.gov/) database was used. DNA sequences were assembled and edited in the program DNA BASER project manager. Based on barcode sequence maximum parsimony and phylogenetic tree construction Was done by MEGA 11 [5]. To improve phylogenetic analysis and removal of alignment noise program Gblocks 0.91b was used [6].

DPPH for estimation of antioxidant activity

Antioxidant activity is determined by the DPPH radical scavenging activity method. Different concentrations of plant extract (5 mg/ml) were taken separately and the volume was make-up by methanol. After that 5 ml of 0.1 millimolar methanolic DPPH solution was added to each test tube. These test tubes were then incubated at room temperature for 20 minutes. After incubation absorbance was taken at the wavelength of 517 nm. Methanol was used as a blank [7].

Antimicrobial activity

Extraction for antimicrobial activity- To extract bioactive components from plant, polar solvent like isopropyl alcohol (Central drug house Pvt. Ltd., India) was used. The maceration extraction procedure was conducted with slight modification. A fresh powdered plant sample was soaked in isopropyl alcohol (1:10) for 48 hours at 25 °C. The filtrate was then collected and dried. The remaining solid residue was again treated with isopropyl alcohol (1:10) and incubated for 48 hours at 50 °C. The modification in the maceration technique will help in the isolation of heat-sensitive bioactive compounds without causing degradation. The combined crude extract was dried at room temperature (25 °C) and stored at -20 °C. To conduct antimicrobial activity agar well diffusion method was adopted. Different concentrations ranging from 10 mg/ml to 1 mg/ml of crude extract was dissolved in DMSO [16-17].

Selected microorganisms- Fungi *Microsporum canis* MTCC 2820, *Trichophyton rubrum* MTCC 296, and *Epidermophyton floccosum* MTCC 7880 were obtained from the Institute of Microbial Technology, Chandigarh, India. While Bacteria, *Bacillus subtilis* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative), fungi *Penicilliuma chrysogeum* and *Candida albicans* were obtained from SMS Medical College, Jaipur, Rajasthan, India.

Isolation and identification of phytochemical

Isolation - The powdered plant sample was taken in an erlenmeyer flask and defatted by a non-polar solvent like n-hexane (1:10). The solid residue was dried at room temperature. The dried solid residue was then macerated in chloroform for 60 minutes at room temperature (1:10). After incubation 0.4 ml of 10%, NH3 was added and kept on a magnetic stirrer. Incubation of mixture was done at room temperature for 30 minutes. After incubation filtrate was collected and dried in a vacuum. The dried filtrate was then dissolved in 100% ethanol and stored at -20 C°. Chloroform+acetone+diethylamine (5+ 4 +1) Solvent system was used for separation. The solvent system is useful in the study of tropane alkaloids [8]. Identification - For identification iodoplatinate reagent, 0.05% (m/v) methanolic DPPH (Bioautography Assay), 50% sulphuric acid, UV 365 and UV 252 was used. Iodoplatinate reagent is use in identification of alkaloid [9]. Methanolic DPPH can help in the identification of antioxidant compounds. 50% sulphuric acid, UV 365, and UV 252 are useful in the Identification at different RF values.

RESULT AND DISCUSSION

Phylogenetic analysis

Phylogenetic analysis was done by sequencing the gene of barcode region matK and ITS. NCBI accession number of sequenced data- Internal transcribed spacer 1, 5.8 ribosomal RNA gene, and internal transcribed spacer 2 (Accession No. MT138451) and MaturaseK (Accession No. OP208801). The length of the sequenced barcode region from matK is 677 bp and ITS 629 bp. The GC content of matk is 31.61 % (Adenine- 211, Thymine – 252, Guanine- 100, Cytosine - 114) and ITS is 50.47 % (Adenine- 154, Thymine - 157, Guanine- 148, Cytosine - 170). After alignment of sequence by clustalW followed by pairwise alignment with the help of neighbor-joining tree, the phylogenetic tree was prepared. The sequence of matK and ITS of *Fagonia laevis* and *Fagonia*

arabica belongs to the same group. Likewise, *Tribulus terrestris* and *Balanites maughamii* are also showing close relationships with both barcode sequences. matK Barcode sequence of *Seetzenia* lanata (Willd.) is showing a close relationship with the group of *Tribulus terrestris* and *Balanites maughamii*, whereas in the case of ITS the sequence of *Seetzenia* lanata (Willd.) is showing more diversity than other plants of Zygophyllaceae (Figure 1).

DPPH radical scavenging assay

DPPH radical scavenging is measured with five different concentrations of plant extract (Graph 1). Comparing it with known antioxidant, the IC50 value of the plant sample is 49.95 while the IC50 value of ascorbic acid is 0.089.

Antimicrobial activity-

Antimicrobial activity in bacteria- In Gram-positive bacteria like *B. subtilis* shows the zone of inhibition at a lower concentration (1 mg/ml) apart from that, no zone of inhibition is visible in Gram-negative bacteria like *P. aeruginosa* (Table-4).

Antifungal activity- Scarcity of antifungal activity in *E. floccosum* and ***A. niger*** is observed with crude extract of *Seetzenia* lanata (Willd.) While in the case of T*. rubrum* zone of inhibition increases simultaneously with the concentration, contrary to that *P. chrysogeum* shows a better zone of inhibition at a lower concentration. Correspondingly *M. canis and C. albicans* show a good zone of inhibition at lower concentrations and no zone at higher concentrations (Table-3).

Thin layer chromatography for identification of phytochemical

An economical and rapid procedure of alkaloid and antioxidant compounds determination in *Seetzenia* lanata (Willd.) is conducted by silica gel-based thin layer chromatography. With the help of three spraying reagents and two wavelengths of ultraviolet radiation secondary metabolite is detected. Resulting in no band detected under visible light and methanolic DPPH (Bioautography Assay), Treatment with ultraviolet radiation 365 nm detected three bands with retardation factor of 0.056(pinkish red), 0.076(sky blue), and 0.097(green). UV 252 nm detected two bands with the retardation factor of 0.056 and 0.27. Treatment with methanol 50% sulphuric acid help in detection of 5 bands showing Rf value of 0.054, 0.076, 0.120, 0.36 and 0.97. One band is visible when the TLC plate is treated with iodoplatinate reagent giving Rf value of 0.054(white band). Overall, one band of Rf value of 0.055 ± 0.001 is visible in UV 365, UV 252, 50% sulphuric acid. Rf value of 0.076 ± 0.001 is visible in 50% sulphuric acid and ultraviolet radiation 365. Although chloroform+ acetone+ diethylamine (5+ 4 + 1) is useful in the separation and identification of nontropane, tropane alkaloids, and alkaloids of an economically important family of Papaveraceae, No Rf value matched with the reference compound [18-19].

Table 1: Temperature in PCR cycles

Primer Name	Initial step		Denaturing step		Annealing step		Extending step	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
ITS	95° C	2 min. 30 sec.	95° C	30 seconds	56° C	30 seconds	72° C	30 seconds
MatK	94° C	3 minutes	94° C	30 seconds	48° C	40 seconds	72° C	1 minute

Table 2: Primers sequence details

Primer Name	Primer Sequence Details
nrITS2-S2-F	5'- ATGCGATACTTGGTGTGAAT-3'
nrITS2-S3-R	5'- GACGCTTCTCCAGACTACAAT-3'
MatK-1RKIM-F	5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'
MatK-3FKIM-R	5'-CGTACAGTACTTTTGTGTTTACGAG-3'

Table – 3: In vitro antifungal activity against the different concentrations of Crude extract.

	T. Rubrum				*M. Canis*				*E. Floccosum*				*P. Chrysogeum*				*C. Albicans*				*A.Niger*			
Conc (mg/ml)	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1
Zone of inhibition [mm]	12	8	6	2	NA	2	4	6	NA	NA	NA	NA	8	8	10	14	NA	NA	4	6	NA	NA	NA	NA

NA- No zone of inhibition.

Table – 4: In vitro antibacterial activity against the different concentrations of Crude extract.

	B. subtilis				*P. aeruginosa*			
Concentrations (mg/ml)	10	5	2.5	1	10	5	2.5	1
Zone of inhibition size [mm]	NA	NA	NA	6	NA	NA	NA	NA

NA- No zone of inhibition.

Graph 1 – DPPH Percentage radical scavenging activity on different concentrations of plant extract.

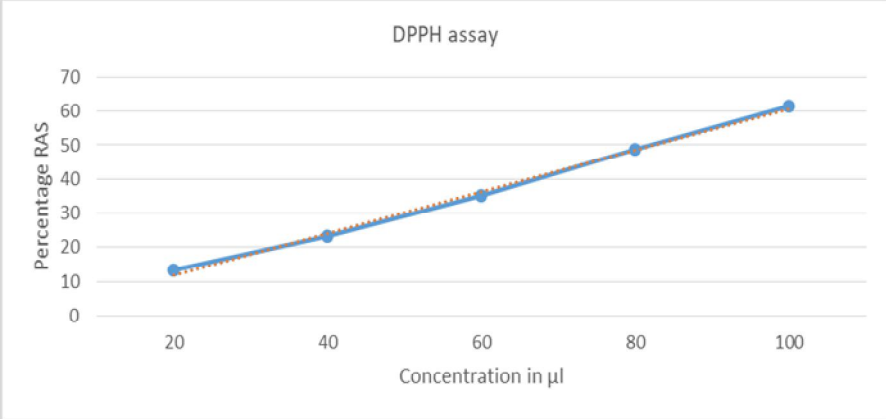
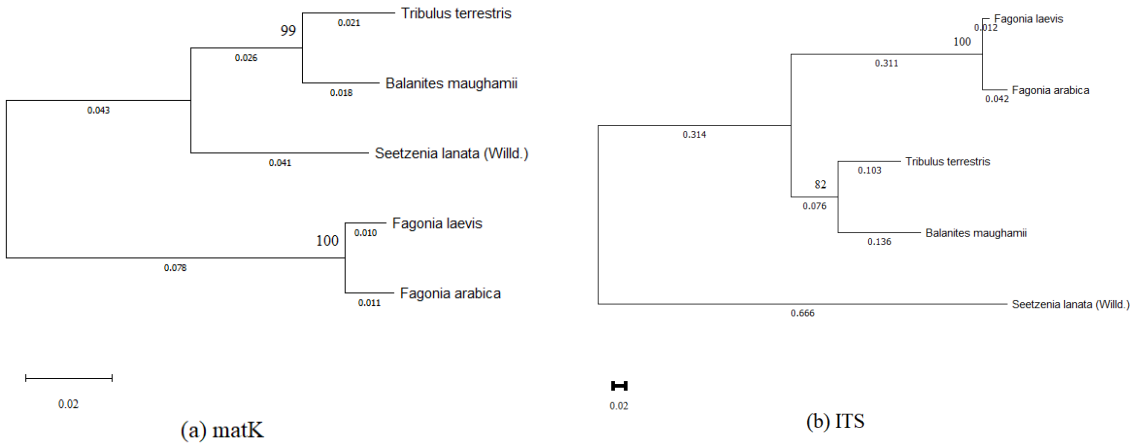


Figure 1 - Phylogenetic tree (analysis among Zygophyllaceae family)



(a) matK (b) ITS

CONCLUSION

To understand the phylogenetic relationship between the Zygophyllaceae family comparative to ITS region, matk is concluded as a good DNA barcode region. Crude extract of plant is showing good antimicrobial activity against *T. rubrum, M. canis, P. chrysogeum* and *C. albicans.* The presence of alkaloid is also identified by iodoplatina reagent.

REFERENCES

1. Christenhusz, M. J., and Byng, J. W. (2016). The number of known plants species in the world and its annual increase. Phytotaxa, 261(3), 201. doi:10.11646/phytotaxa.261.3.1
2. Bhandari,M.M.(1990) Flora of the Indian Desert, p. 79.
3. Yu, J., Wu, X., Liu, C., Newmaster, S., Ragupathy, S., and Kress, W. J. (2021). Progress in the use of DNA barcodes in the identification and classification of medicinal plants. Ecotoxicology and Environmental Safety, 208, 111691. doi:10.1016/j.ecoenv.2020.111691
4. Mahdi-Pour, B., Jothy, S. L., Latha, L. Y., Chen, Y., and Sasidharan, S. (2012). Antioxidant activity of methanol extracts of different parts of lantana Camara. Asian Pacific Journal of Tropical Biomedicine, 2(12), 960-965. doi:10.1016/s2221-1691(13)60007-6
5. Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5), 1792-1797. doi:10.1093/nar/gkh340
6. Talavera, G., and Castresana, J. (2007). Improvement of Phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systematic Biology, 56(4): 564-577. doi:10.1080/10635150701472164
7. Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181(4617), 1199-1200. doi:10.1038/1811199a0
8. Stahl,E.(1969). Thin-Layer Chromatography A Laboratory Handbook, 2 ed.,432. doi: 10.1007/978-3-642-88488-7
9. Baerheim-Svendsen, A. and Verpoorte, v. (1983) Chromatography of alkaloids part a: Thin-layer chromatography., 23(a): 20.
10. Okeke, M., Iroegbu, C., Eze, E., Okoli, A., & Esimone, C. (2001). Evaluation of extracts of the root of Landolphia owerrience for antibacterial activity. Journal of Ethnopharmacology, 78(2-3), 119-127. doi:10.1016/s0378-8741(01)00307-5
11. Magaldi, S., Mata-Essayag, S., Hartung de Capriles, C., Perez, C., Colella, M., Olaizola, C., & Ontiveros, Y. (2004). Well diffusion for antifungal susceptibility testing. International Journal of Infectious Diseases, 8(1), 39-45. doi:10.1016/j.ijid.2003.03.002
12. Glavind, J., & Holmer, G. (1967).Thin-Layer Chromatographic Determination of Antioxidants α,α' Diphenyl-β-picrylhydrazy. Journal of the American Oil Chemists' Society, 44(9), 539-542. doi:10.1007/bf02679243
13. Heftmann, E., Ko, S., & Bennett, R. D. (1966). Response of steroids to sulfuric acid in thin-layer chromatography. Journal of Chromatography A, 21, 490-494. doi:10.1016/s0021-9673(01)91347-0
14. Chauhan, S. S. (2003). Desertification control and management of land degradation in the Thar desert of India. The Environmentalist, 23(3), 219-227. doi:10.1023/b:envr.0000017366.67642.79
15. Sinha,R.K., Bhatia, S. and VishnoI, R. (1996)"Desertification control and rangeland management in the Thar desert of India,"RALA Report No. 200: 115–123
16. Udensi, O., Ita, E., Ikpeme, E., Ubi, G., & Emeagi, L. (2017). Sequence analysis of Maturase K (matK): A chloroplast-encoding gene in some selected pulses. Global Journal of Pure and Applied Sciences, 23(2), 213. doi:10.4314/gjpas.v23i2.2
17. Zhang, D., Jiang, B., Duan, L., & Zhou, N. (2016). Internal transcribed spacer (Its), an ideal DNA barcode for species discrimination in crawfurdia wall. (Gentianaceae). African Journal of Traditional, Complementary and Alternative medicines, 13(6), 101-106. doi:10.21010/ajtcam.v13i6.15
18. Edreva, A. (2005). Generation and scavenging of reactive oxygen species in chloroplasts: A submolecular approach. Agriculture, Ecosystems & Environment, 106(2-3), 119-133. doi:10.1016/j.agee.2004.10.022
19. Gupta, S. C., Hevia, D., Patchva, S., Park, B., Koh, W., & Aggarwal, B. B. (2012). Upsides and downsides of reactive oxygen species for cancer: The roles of reactive oxygen species in Tumorigenesis, prevention, and therapy. Antioxidants & Redox Signaling, 16(11), 1295-1322. doi:10.1089/ars.2011.4414
20. Shah, G. L. (1978) Flora of Gujarat state part 1, Sardar Patel University,:139.
21. Pandey,R. P.(1987) Zygophyllaceae," Flora of india series 2(2), p. 167.