Materials and Methods

CHAPTER - IV

4.1 Standard Literature Used for Identification and Herbarium Consultation:

An extensive and critical survey of concerned literature on pteridophyte and gymnosperm flora of Gujarat state has been made. Comprehensive information on pteridophyte and gymnosperm of the state and their occurrence, distribution, status and diagnostic features etc., were collected through referring to various research publications, state and national floras, monographs and books. Electronic sources *viz*., The Biodiversity Heritage Library (https://www.biodiversitylibrary.org/); eFloras (http://efloras.org); International Plant Names Index (http://www.ipni.org); The Plant List (http://www.theplantlist.org); Tropicos (http://www.tropicos.org); Plants of the World, Kew Science (http://www.plantsoftheworldonline.org/); JSTOR Global Plants (https://plants.jstor.org); Ferns of Thailand, Laos and Cambodia (http://rbg-web2.rbge.org.uk/thaiferns/) and Checklist of Ferns and Lycophytes of the World (https://worldplants.webarchiv.kit.edu/ferns/) were also used for retrieving of species identification, its distribution and ecological studies. Critical notes on each species were prepared by consulting recent literature and various specimen records deposited in different herbaria *viz*., BARO (Department of Botany, The M. S. University of Baroda, Vadodara, Gujarat), BSJO (BSI, Arid and Semi-arid regional centre, Jodhpur, Rajasthan), BSI (BSI, Western Regional Centre, Pune, Maharashtra), BLAT (Blatter's Herbarium, Mumbai, Maharashtra) and CAL (Central National Herbarium, Kolkata, West Bengal). Authentic virtual herbarium sites were also used for identification of species *viz*., Kew Herbarium Catalogue (https://apps.kew.org/herbcat/navigator.do), JSTOR (https://plants.jstor.org/), NYBG Steere Herbarium (http://sweetgum.nybg.org/science/vh/specimen-list/) and Herbarium JCB (http://florakarnataka.ces.iisc.ac.in/hjcb2/index.php).

4.2 Field Work, Specimen Collection and Processing:

Based on available literature and herbarium information, several explorative field survey trips of short and long duration were undertaken to various localities of Gujarat state for seven years (2013-2020). Every possible area of the state which supports the growth of pteridophyte and gymnosperm in the forest region, wetlands, streams, natural ponds and desert area were surveyed habitually for successive seven years. These explorative tours were arranged in the monsoon and post-monsoon seasons when their growth and occurrence is abundant and luxuriant. For the period of field exploration, observations were made on life form, ecology, distribution and occurrence and variations

in species and threat encountered by the species in their natural habitat. In the field, minute details of the specimens of both pteridophytes and gymnosperms such as habit, habitat (terrestrial, lithophyte, epiphyte and aquatic), root, stem, nature and size of the frond, rhizome, stipe, rachis, pinnatification of the lamina, scales on the rhizome, stipe, rachis and costa, arrangement of sori, cones, strobilus etc., were also recorded. Saplings of some species were also collected from the field and grown in the botanical garden and arboretum of the Maharaja Sayajirao University of Baroda, Vadodara for further studies. Critical micro- and macro-morphological observations of each specimen have been made in the field and diagnostic features were recorded in the field note, collection numbers and date were also given to each specimen. For epiphytic ferns, the information about host species was recorded in the field itself and field photographs were clicked in their natural habitat with the help of digital camera (Canon SLR 1200D). Specimens were also collected and pressed in field press using blotting paper, while, rest of the samples were packed in sterile polyethene bags with dried silica beads for the molecular studies in the laboratory. Fertile specimens were used to study gross micro-morphology and spore characteristics by using Leica MSZE6 Stereomicroscope and Leica DME 2000 compound microscope. For microscopic observations, scales on various parts such as rhizome, stipe, rachis, costa etc., were excised and soaked in the distilled water for 20-30 min., whereas the morphology of spores were examined by collecting from the fertile sporangium and they were soaked in 50% glycerin solution and observed under a microscope by mounting on clean slide that was covered with a coverslip. Samples collected for molecular studies were stored in deep freeze at -4^0C.

4.3 Identification of Collected Specimens:

The identification, confirmation and nomenclature of the collected specimens were done by referring standard literature, state and national floras, monographs and books (Beddome 1873, 1892; Blatter & d'Almeida 1922; Panigrahi & Dixit 1969a-c; Manickam & Irudayaraj 1992; Vasudeva & Bir 1994; Khullar 1994, 2000; Fraser-Jenkins *et al*. 1997, 2008b, 2015, 2017, 2018a, 2020; Ghosh *et al*. 2004; Kholia 2010a, 2014b; Singh & Srivastava 2013). Tropicos, IPNI, The Plant list and World Ferns have been followed for species nomenclature. Indented keys have been prepared for easy identification of genera and species. In the present comprehensive research work, classification proposed by PPG-I (2016) and Singh & Srivastava (2013) has been followed for family and genus level status of pteridophytes and gymnosperms respectively.

4.4 Molecular Identification:

4.4.1 DNA Isolation

Specimens stored in deep freeze were brought to room temperature. Silica beads added in polythene bags at the time of collection were removed from the samples and green healthy leaves or fronds were washed thoroughly in sterile distilled water 2-3 times repeatedly. Subsequently, the samples were surface sterilized by washing with 70% ethanol followed by 2-3 times rinsing in sterile distilled water. Samples weighing 50 mg were crushed and homogenized in liquid nitrogen with a pinch of PVP (Polyvinylpyrrolidone) by using mortar and pestle. The homogenated fine powder was collected in a sterile 2 ml microcentrifuge tube and processed for DNA isolation. Some of the homogenated samples were also collected in a sterile 2 ml microfuge tube and stored at -4°C in deep freeze for future study (if required). Isolation of total genomic DNA from crushed samples was carried out by using Plant/Fungi DNA isolation kit (Sigma Aldrich Cat# E5038) following manufacturer protocol or manually by CTAB method as described by Doyle & Doyle (1987). Some of the specimens that failed in isolation of DNA by Plant/Fungi DNA isolation kit, in such samples, a stored homogenate were subjected for isolation of total genomic DNA by modified CTAB method.

4.4.2 Modified CTAB method for DNA Isolation

- The homogenate sample was mixed thoroughly in 500 µL Buffer (B) containing 100 mM Tris-Cl (PH: 8), 50 mM EDTA (PH: 8), 1 M NaCl, 0.5 M Sucrose, 2% Triton X-100 and 0.2 % β-mercaptoethanol and add 10 µL of RNaseA.
- The mixture was then incubated at 65°C for 30 minutes in a boiling water bath with occasional mixing.
- After incubation, the mixtures were centrifuged for 10 at 10,000 rpm and then decant the supernatant.
- 500 µL Buffer (A) comprising 100 mM Tris-Cl (PH: 8), 50 mM EDTA (PH: 8), 3.5 M NaCl, 2% CTAB, 1% PVP, 5% SDS and 1% β-mercaptoethanol was added into Eppendorf tube having pellet and mixed well.
- The mixture was again incubated in a boiling water bath at 65°C for 30 minutes with occasional mixing.
- Subsequently, the samples were cooled at room temperature and chloroform/isoamyl alcohol (24:1) 500 µL was added to the tubes.

- The sample was homogenized by gentle shaking inversely for 5-10 minutes.
- The clear two phases were separated by centrifuge at 8,000 rpm for 8 minutes.
- The upper layer or supernatant was transferred into new 2 ml Eppendorf tube. Then add equal volume of CI and shack in inversely for 5-10 min. and centrifuge at 8,000 rpm for 8 min.
- Take supernatant in the new 2 mL Eppendorf tube and add the two-thirds amount of cold isopropanol and one-third amount of 5 M NaCl. Mixed well and was incubated overnight at -4°C.
- On the next day, the mixture was centrifuged at 10,000 rpm for 10 min to settle down a pellet of the genomic DNA. The supernatant was discarded and pelleted DNA was washed with chilled 70% ethanol two times by gentle mixing followed by centrifugation at 10,000 rpm for 10 min.
- The supernatant was discarded and the pellet was air-dried at room temperature.
- Subsequently, the pellet was dissolved in 100 µL TE buffer containing 10 mM Tris-HCl (pH: 8.0) and 1 mM EDTA (pH: 8.0).
- The isolated DNA was stored at -4°C and used for further analysis.
- Agarose gel electrophoresis was performed to visualize DNA on 0.8 % Agarose gel.

4.4.3 PCR Amplification

The PCR amplification was bring by using 2X final concentration of dyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, Missouri, USA) or DreamTaq Green PCR Master Mix (Thermo Scientific; Cat# K1081), primers, template DNA (50 ng/µl) and distilled water in PCR thermal cycler (Applied Biosystems Veriti® thermo-cycler). PCR reaction mixture, condition and different primers of chloroplast and mitochondrial region used for successful DNA amplification are tabulated in table 1-3. Primers were synthesized from Eurofins genomics Pvt. Ltd. Bangalore. The PCR products were visualized on electrophoresed 2% Agarose gel using TBE buffer.

Table 1: PCR amplification reaction mixture.

Sr. No.	Reagent	Reaction Volume	
1	2x DreamTaq Green PCR Master Mix (Cat# K1081)	10 µL	
2	Forward primer (10 pM)	1 µL	
3	Reverse primer (10 pM)	1 µL	
4	DNA template (~50ng/µL)	1 µL	2 µL
5	Sterile double distilled water (Nuclease free)	7 µL	6 µL

Total Reaction Volume	20 µL

Table 2: PCR primers and conditions.

Region	Primer	Stage - I (1 Cycle)	Stage - II (35 Cycles)			Stage - III (1 Cycle)
		Pre-denaturation	Denaturation	Annealing	Extension	Final Extension
rbcL	rbcLa-F rbcLa-R	94.0°C 4 min.	94.0°C 30 sec.	54.0°C 30 sec.	72.0°C 1:30 min.	72.0°C 10:00 min.
trnFE	trnF-F trnE-R	94.0°C 4 min.	94.0°C 30 sec.	54.0°C 30 sec.	72.0°C 1:30 min.	72.0°C 10:00 min.
trnLF	trnLF-F trnLF-R	94.0°C 4 min.	94.0°C 30 sec.	55.0°C 30 sec.	72.0°C 1:30 min.	72.0°C 10:00 min.
psb-trnHf	psbA3_f trnHf_05	94.0°C 4 min.	94.0°C 30 sec.	54.0°C 30 sec.	72.0°C 1:30 min.	72.0°C 10:00 min.
ITS2	ITS2- ITS4	94.0°C 4 min.	94.0°C 30 sec.	56.0°C 30 sec.	72.0°C 1:30 min.	72.0°C 10:00 min.

Table 3: List of chloroplast and nuclear region primers were synthesized.

Region	**Primers**	**Sequence (5' → 3')**	**Reference**
Chloroplast	rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	Levin (2003)
	rbcLa-R	GTAAAATCAAGTCCACCRCG	Kress & Erickson (2007)
Chloroplast	trnF-F	ATTTGAACTGGTGACACGAG	Asif & Cannon (2005)
	trnE-R	GGTTCAAGTCCCTCTATCCC	Asif & Cannon (2005)
Chloroplast	trnLF-F	CGAAATCGGTAGACGCTACG	Taberlet *et al*. (1991)
	trnLF-R	ATTTGAACTGGTGACACGAG	Taberlet *et al.* (1991)
Chloroplast	psbA3_f	GTTATGCATGAACGTAATGCTC	Sang *et al*. (1997)
	trnHf_05	CGCGCATGGTGGATTCACAATCC	Tate & Simpson (2003)
Nuclear	ITS2-S2F	ATGCGATACTTGGTGTGAAT	Chen *et al*. (2010)
	ITS4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)

4.4.4 PCR Purification and Sequencing

The amplified PCR products were purified using PurelinkTM Quick PCR Purification kit (Thermo Fisher Scientific; Cat# K310001). Further, purified PCR products were sent for sequencing to Eurofins Genomics India Pvt. Ltd., Bangalore.

4.4.5 DNA Sequence Analysis and Submission to BOLD Systems and NCBI

Sequences obtained from Eurofins Genomics India Pvt. Ltd. were subjected for analysis in BioEdit and/or Finch TV. Forward and reverse sequence was assembled and contig in BioEdit. Contig sequences were subjected for nucleotide BLAST analysis on the GenBank database (blast.ncbi.nlm.nih.gov) for identification of the species. Later, identification was done by matching up to 99% base-pair of the sequence obtained to the closest available reference sequences of GeneBank database. The sequences which showed significant match in with NCBI Database were submitted to the BOLD Systems (http://www.boldsystems.org/) and NCBI (National Center for Biotechnology Information) by using BankIt (https://www.ncbi.nlm.nih.gov/WebSub/).

4.5. Confirmation and Authentication of Specimens:

Collected specimens were identified using standard references and literature followed by experts in the field of pteridophytes and gymnosperms were consulted by sending the good quality photo plates containing required detail were sent as an email attachment or scanned/photographs of herbaria along with specific characteristics of a plant to Prof. S. P. Khullar (Chandigarh) and Mr. C. R. Fraser-Jenkins (Portugal) for identification and confirmation of their identity. Specimens were also carried in seminar/symposia meetings to consult the experts. Several other field experts were also consulted for the identification, confirmation and authentication of collected specimens *viz*., Prof. H. K. Goswami (Bhopal), Prof. N. Punetha (Pithoragarh), Dr. B. S. Kholia (Dehradun), Dr. A. Benniamin (Pune), Prof. P. L. Uniyal (Delhi), Dr. Sachin M. Patil (Vadodara), Dr. Jaydeep Mujumdar (Burdwan), Dr. Nirupama Goswami (Burdwan), Dr. P. Vijaykanth (Krishnagiri) and Dr. Bhukani (Uttarakhand).

4.6 Herbarium Preparation and Submission:

Field pressed samples were dried using blotting sheets and old newspaper for the preparation of herbarium. Dried specimens were poisoned using mercuric chloride (0.05% w/v) dissolved in ethanol and air-dried under the fan. Dried and poisoned specimens were mounted on herbarium sheets by stitching with thread or masking tape (Gummed lined tape). Herbariums are deposited in BARO herbarium of the Botany Department, The M. S. University of Baroda - Vadodara (BARO); Arid and Semi-arid

regional center, Jodhpur, Rajasthan (BSJO); Western Regional Centre, Pune, Maharashtra (BSI); The Blatter Herbarium, Mumbai, Maharashtra (BLAT) and Central National Herbarium, Kolkata, West Bengal (CAL) and certificate with the accession number of each species were procured (Annexure 1-4, Appendix 1). Herbarium specimens were arranged by following the classification of PPG-I (2016) and Singh & Srivastava (2013) for family, genus and species of pteridophyte and gymnosperm respectively.

4.7 Species Distribution Mapping:

The geographical coordinates of collected specimens were recorded by using a Garmin GPS or Mobile application (GPS Status). For distribution of each species in the state, Google Earth Software was used by applying GPS coordinates and KMZ format files were created. A digital image administrative Gujarat state map including 33 districts was downloaded from the internet and different climatic zones of the state were colored by editing in Adobe Photoshop 7.0 by using standard reference. State map was Georeferenced in the QGIS software by overlapping authentic toposheet of the state available on the official website of the Indian government (http://soinakshe.uk.gov.in/). This Georeferenced file was used for the distribution of each species in all the districts along with the ecological zones of the state. The distribution map for each pteridophyte and gymnosperm species in Gujarat state were prepared by using QGIS. For the distribution of pteridophytes and gymnosperms in the different bio-geographical zones of Gujarat, a map was downloaded from Wildlife Institute of India, IT and RS & GIS Cell - 2015 (Anonymous 2021d). This map was Georeferenced by overlapping the authenticated points available in the image with the help of QGIS Software.

4.8 IUCN Red-list Assessment, Categories and Criteria:

The IUCN Red List of Threatened Species is renowned as the most comprehensive and objective global approach for assessing the conservation status of the plant regional, state and globally. It is a widely-recognized tool for identifying threatened species in a particular area and suggest a powerful method to identify the importance of sites for protection by providing a piece of information on the conservation status of species in the wild (Rodrigues *et al*. 2006). In 1994 and 2001, IUCN Red List categories and criteria were deliberate the assessment of extinction threat of the species at the global level. In 2012, IUCN published guidelines for the application of the IUCN Red List Criteria at Regional and National Levels (version 4.0). For the present comprehensive study, mature and healthy individuals were counted in each locality for

determining the status of the species. Direct observations were made to recognize the possible and actual threats to the species population at the regional level.

4.8.1 IUCN Red List Categories (version 3.1)

IUCN (2016), classified nine clearly defined categories for the taxa in the world in three demarcated group *viz*., Extinct category [Extinct (EX), Extinct in the Wild (EW)], Threatened category [Critically Endangered (CR), Endangered (EN), Vulnerable (VU)] and Lower risk category [Near Threatened (NT), Least Concern (LC), Data Deficient (DD) and Not Evaluated (NE)].

4.8.2 IUCN Red List Criteria (version 3.1)

IUCN (2016) listed five quantitative criteria, which govern whether a taxon is threatened or not and if threatened, the category of the threat it belongs in (Critically Endangered, Endangered or Vulnerable). Criteria are largely based on population size and its decline, geographical ranges and quantitative analysis. Maximum criteria include sub-criteria that must be used to justify additional specifically citation of a taxon under a specific category.

Five criteria are

A. Declining population (past, present and/or projected)

B. Geographic range size, and fragmentation, decline or fluctuations

C. Small population size and fragmentation, decline, or fluctuations

D. Very small population or very restricted distribution

E. Quantitative analysis of extinction risk (e.g., Population Viability Analysis)

In order to list taxa in some of categories of threat, at least one of the criteria A, B, C, D, or E should be met.

4.8.3 Regional Level Conservation Assessment of the Taxon

IUCN (2012) Version 4.0 categorized 11 possible categories for regional level assessments (continent, country, state or province) of taxon which is the same as the global assessment (Fig. 3). However, there are three exceptions or adjustments for the regional assessment of taxon.

Firstly, the taxa extinct in region (state) but extant in other parts of the world, continents or country should be classified as ‘Regionally Extinct (RE)’. Furthermore, a taxon is Regionally Extinct, when no reasonable uncertainty that the last individual accomplished of reproduction within the region has died or disappeared from the region or, in the case of a former visiting taxon; individuals no longer visit the region. Listing of the taxon as ‘Regionally Extinct’ requires comprehensive exploration and survey in all

known or possible habitats of the particular region. However, there is no such taxon present in the state.

Second, the category of 'Extinct in the Wild (EW)' would be assigned only to the taxa that are extinct in the wild across their whole natural range, including the region, but that are extant in cultivation, in captivity, or as naturalized population (or populations) outside the past range. If a taxon is (globally) extinct in the Wild, but extant as naturalized population in the region, the regional population would not be assessed according to IUCN criteria, but would still be measured of conservation importance and conserved as a relic of a taxon, which is extinct in the Wild. It may also be considered an important source of individuals for reintroduction efforts within its natural range. There is no such taxon present in the state.

Third, taxa not eligible for assessment at the regional level (mainly introduced taxa and vagrants) should be assigned the category 'Not Applicable (NA)'.

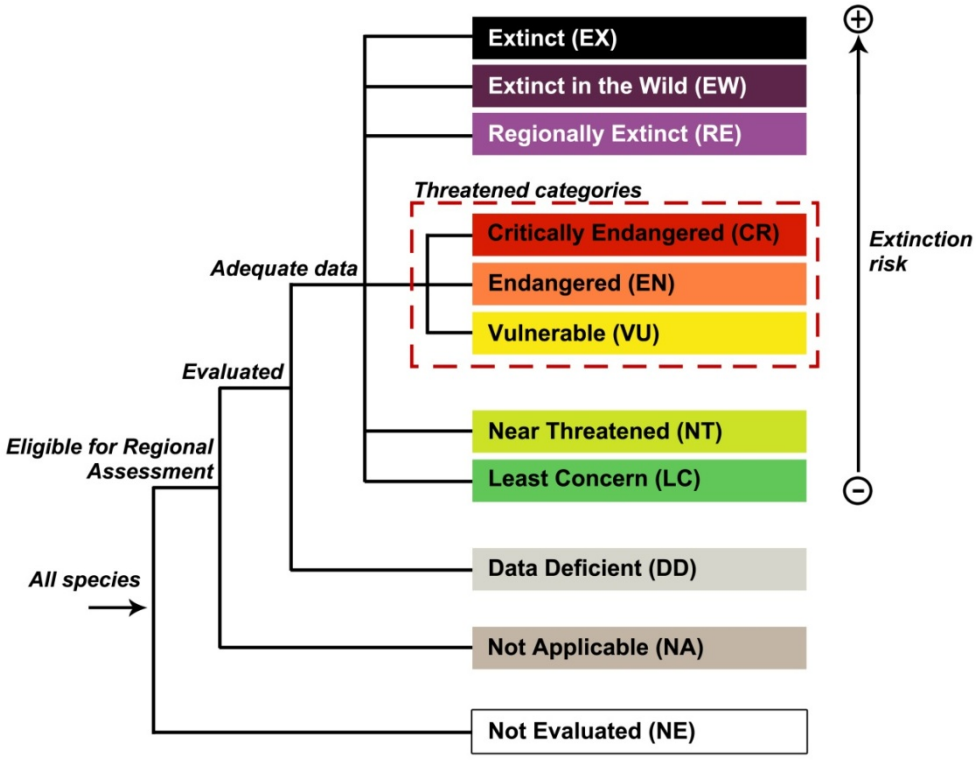

Figure 3: Structure of the categories used at the regional level (IUCN 2012).

In the present study, a brief description of the IUCN categories B & D (except A, C and E) were used in the regional assessment (Gujarat state) of pteridophytes and gymnosperm of Gujarat (Table. 4). Criteria A, C and E were not used for the present

assessment because there is no data on the defined rate of population decline coupled with the small population size, etc.

Table 4: IUCN Red-list Criteria (B & D) used to evaluate pteridophyte and gymnosperm of Gujarat state if any belongs in an IUCN red-list threatened category.

B. Geographic range in the form of either B1 (extent of occurrence) AND/OR B2 (area of occupancy)			
	Critically Endangered	**Endangered**	**Vulnerable**
B1. The extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²
AND at least 2 of the following 3 conditions:			
(a) Severely fragmented OR Number of locations	= 1	≤ 5	≤ 10
(b) Continuing decline observed, estimated, inferred or projected in any of **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** area, extent and/or quality of habitat; **(iv)** number of locations or subpopulations; **(v)** number of mature individuals			
(c) Extreme fluctuations in any of **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** number of locations or subpopulations; **(iv)** number of mature individuals			
D. Very small or restricted population			
	Critically Endangered	**Endangered**	**Vulnerable**
D1. Number of mature individuals	< 50	< 250	**D1.** < 1,000
D2. Only applies to the VU category Restricted area of occupancy or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.	-	-	**D2.** typically: AOO < 20 km² or number of locations ≤ 5

4.8.4 Calculation of Area of Occupancy (AOO) and Extent of Occurrence (EOO)

According to IUCN (2016), a taxon is measured as in the geographic range of size under two parameters *viz*., EOO (Extent of Occurrence) and AOO (Area of

Occupancy). The extent of occurrence (EOO) of any taxon is measured by the smallest polygon in which no internal angle exceeds 1800 and contains all sites of occurrence. Area of Occupancy (AOO) is the area occupied by taxon, excluding cases of destitution, at a scale appropriate to the taxon.

These two measures are the foundation of the criteria 'B' of the IUCN Red-List system (IUCN 2016). EOO provides information on the overall geographical spread in the state. While, AOO provides information of the area of suitable habitat. Both EOO and AOO were calculated using the Geospatial Conservation Assessment Tool (GeoCAT; http://geocat.kew.org/), which is developed by Royal Botanic Gardens, Kew. All the occurrence data of each species was prepared in a separate spreadsheet that includes relevant GPS points. These data were directly imported to the GeoCAT tools in CSV file format. Based on the location points, the extent of occurrence (EOO) and area of occupancy (AOO) values are instantly calculated and the values were compared with the onsets set in the IUCN Criteria (2016) for listing a species in the red-list (if any?).

4.8.5 Threats to the Species (If Any?)

Various threats to the taxon were also observed during the field visits, which include habitat destruction, fragmentation and modification of natural habitats, tourism activities, mining and stone excavating, illegal collection for medicinal purpose, grazing, fire, invasive species and natural disasters.