Assessment of Genetic Diversity, Population Structure and Phytochemical Variations in *Polygonatum Cirrhifolium* (Wall.) Royle - An Endangered Medicinal Herb

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Abstract

Polygonatum cirrhifolium is an important medicinal herb of family Asparagaceae used to cure several ailments. Its rhizome forms an important ingredient of "Chyavanprash" which is identified for its rejuvenating properties. However, *P. cirrhifolium* is least explored scientifically and systematically till date. Therefore, in this study genetic diversity and phytochemical variations along with antimutagenic activity of *P. cirrhifolium* populations was evaluated. Antimutagenic activity varied remarkably ($p < 0.05$) having Gagar population with significantly ($p < 0.05$) higher DNA recovery (84.95%) percentage. Higher genetic diversity (H_e) was recorded among populations using RAPD (H_e , 0.30-0.36) and ISSR (0.25-0.38) markers. High intra and low inter population variations were recorded in the species using both kinds of markers. Phenolics ($p < 0.05$; $r = 0.924$); tannins ($p < 0.05$; $r = 0.897$) and DNA damage inhibition efficiency displayed a highly positive correlation with genetic diversity (estimated using ISSR markers). The population structure analysis of *P. cirrhifolium* revealed that the greatest value of the K was 3 for studied populations. Gene flow among studied populations was found sufficient to encounter genetic erosion in the species. Therefore, it can be recommended that the populations with higher ingredient and genetic diversity can be utilized for conservation priority and management plan of this species.

Introduction

Polygonatum Mill. (Asparagaceae) is a genus of perennial herbs, owing to its wide array of health benefits the species of this genus has been used traditionally. The genus comprises of 71 species of which 37 species are reported for medicinal value (Zhao et al. 2018). Most *Polygonatum* species comprises global curative properties and hence, consists of positions in Indian Ayurveda, traditional Chinese medicines, different pharmacopoeias, etc. *Polygonatum* herbs are rich source of diosgenin, polysaccharides, steroidal saponins, homoisoflavanones, etc. (Liu et al. 2011; Khan et al. 2012; Wang et al. 2019) and hence, efficient as cardio-tonic (Hirai et al. 1997), anti-inflammatory (Khan et al. 2013), antispasmodic (Khan et al. 2016), antimutagenic (Suyal et al. 2019a) etc. Most of the important *Polygonatum* species lacks phytochemical, pharmacological and genetic diversity researches. One such vital herb is *Polygonatum cirrhifolium* (Wall.) Royle generally recognized as Mahameda. The species is disseminated in the Himalayas from India, Bhutan, Nepal and China, to Pakistan (www.tropicos.org). Diverse plant parts of species like leaf, seeds, and rhizomes are utilized to treat kidney, rheumatism, and dermatological disorders. Rhizomes forms primary raw material of the Astavarga formulation, which is utilized in seminal weakness and as a cure for rakta, vata and pitta doshas (Balakrishna et al. 2012). The rhizome, stems and leaves of *P. cirrhifolium* are rich in bioactive sources such as steroidal saponins, flavonoids, anthraquinones, and their glycosides, coumarins, carbohydrates, etc. In addition, proteins, amino acids and polyphenols are also recorded from roots and lactones in stems and leaves (Wang et al. 2006).

P. cirrhifolium is adapted in several ecological habitats having prevalent variations in environmental conditions which ultimately affect the superiority and efficacy of the ultimate produce (Suyal et al. 2019a). Thus, evaluation of quality resources of *P. cirrhifolium* is essentially required. According to local and unofficial reports huge amount of plant rhizome is collected annually from the Himalayan area due to species demand in domestic and global market and hence, depleted the species germplasm. Few reports are existing on diversity and distribution (Suyal et al. 2019b), propagation protocols (Latoo et al. 2005) and pharmacological activity (Xu et al. 2017) of *P. cirrhifolium*. However, none of the studies so far reported the genetic diversity, phytochemical, and anti-mutagenic property of the species. Studies on these aspects are necessary for elite identification and managing the species. Studies of genetic diversity are getting popularity across the world for setting priorities of conservation, especially to introduce the species in its natural habitats. Moreover, only few reports are available on the genetic multiplicity evaluation of herbal species from Himalayan region (Jugran et al. 2013; Giri et al. 2016; Fang et al. 2018; Ray et al. 2019). This signifies an urgent need to quantify the genetic diversity among the populations of threatened and endemic medicinal plants species towards predicting the future persistence. Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR) markers are among such markers that are extensively employed for assessing genetic diversity and population structure studies of several plant species due to their cost effectiveness, robustness and high reproducibility (Fang et al. 2018). However, no prior information is available on the genetic diversity analysis and antimutagenic activity of *P. cirrhifolium*. Therefore, the major objectives of this investigation is to (i) characterize the genetic structure and differentiation of the studied populations, (ii) examine phytochemical, antioxidant and DNA damage inhibition efficiency of selected populations, and (iii) to establish relationship among phytochemicals, antioxidant properties and molecular attributes.

Material And Methods

Exploration and collection of plant material

Extensive field investigation was carried in Kumaun region (28°44' to 30°49'N latitude & 78°45' to 81°05'E longitude) of IHR (Indian Himalayan Region) where *P. cirrhifolium* is predominantly found. A total of five different naturally occurring populations were selected and sampled randomly. Representative leaves of samples from each population were collected, placed in the coarse silica gel and brought in the laboratory for further analysis. Rhizomes of *P. cirrhifolium* were utilized to measure the phytochemicals, DNA damage preventive or antimutagenic and antioxidant activities. The experiments in the current study were conducted in the CBCM (Centre for Biodiversity Conservation and Management), Govind Ballabh Pant National Institute of Himalayan Environment (GBP-NIHE). Details of geographical coordinates and site characteristics were also recorded (Table 1).

Preparation of extract

Rhizomes of each population was separated and thoroughly washed with tap water followed by ultra pure water (Rions India Lab Water System, India). Rhizomes were dried at 40°C in a hot air oven and grounded into fine powder. Dried powder material (1 g) was added in 25 mL of methanol (80% v/v) and the suspension was incubated in water bath at 60°C for 1 h. Further the suspension was kept for incubation for 24 h at 25°C and sonicated at 22°C for 10 minutes (Model - ANIS 09001, Toshiba, Delhi, India). After filtering the solution it was centrifuged at 22±1°C at 10,000 rpm for up to 15 minutes. Thereafter, the supernatants was collected in DW and kept at 4°C to use within 2 days.

Determination of total phenols

Methanol extract derived from rhizome of the species was used to measure total phenolic content by Folin- Ciocalteu's colorimetric method (Singleton and Rossi 1965). Methanolic extract (0.50 ml) was diluted with dH₂O (4.50 ml) followed by addition of 0.50 ml Folin–Ciocalteu's reagent. The mixture was kept at temperature for reaction up to 5 minutes. Thereafter, 5.0 ml of sodium carbonate (7%) was used for neutralizing the reaction and the solution was placed in dark at 25°C for 90 minutes. UV–vis spectrophotometer was used to record the absorbance of the solution at 765 nm using. The amount of total phenolics present in the extract was calculated as mg GAE (gallic acid equivalent) per g DW.

Total tannins estimation

Total tannins in the plant methanol extract were measured by applying the method developed by Nwinuka et al. (2005) with slight modification. 5 ml of methanol extract was mixed with 0.5 ml of Folin-Dennis reagent. The mixture was than saturated with 7% of sodium carbonate (1.0 ml) followed by placing the solution in dark at 22±1°C for 20 min. The absorbance of the solution was noted at 700 nm and the results were calculated as mg TAE (tannic acid equivalent) per g of DW.

Determination of total flavonoids and flavonol

Aluminium chloride colorimetric method was used to measure total flavonoids in the methanolic extract of the species (Chang et al. 2002). Rhizome extract (0.50 ml) was thinned with dH₂O (1.5 ml) and aluminium chloride (0.50 ml of 10% w/v) along with addition of 1 M potassium acetate (0.10 ml) and dH₂O (2.80 ml). The absorbance of the mixture was recorded at 415 nm after 30 minutes incubation at room temperature results were expressed in mg QE (quercetin) equivalent/ g of DW.

assay developed by Kumaran and Kaunakaran (2007) was applied to estimate total flavonol content in the methanol extract of the species rhizomes. Briefly, extract (2.0 ml), 2.0 ml 2% ethanolic aluminium chloride (w/v) and sodium acetate solution (3.0 ml) were added. The mixture was placed at room temperature for reaction for 2.5 h at 20°C. The absorbance was recorded at 440 nm and the results were calculated as mg QE (quercetin equivalent) / g dw.

Antioxidant activity

Radical scavenging activity (ABTS assay)

Antioxidant property in the rhizome of *P. cirrhifolium* was determined by ABTS method (Cai et al., 2004). ABTS cation (ABTS•+) was produced by mixing potassium persulfate (2.45µM) with ABTS salt (7.0µM) followed by placing solution in dark for 16 h at 23°C. ABTS•+ solution was thinned with ethanol (80%,v/v) to obtain an absorbance of 0.7±0.005 at 734 nm. ABTS•+ solution (3.90 ml) was mixed with methanolic extract (0.10 ml) and vortexes to mix properly. The reaction mixture was placed in dark for 6 min at 25°C followed by noting absorbance at 734 nm. Samples were diluted with methanol (80%, v/v) to obtain 20–80% reduction in absorbance as compared to blank that was prepared with 0.1 ml methanol (80%, v/v). The results were shown as milli mole ascorbic acid equivalent (mM AAE) per 100 g dry weight.

Radical scavenging activity (DPPH assay)

Slightly modified DPPH method developed by Brand-Williams et al. (1995) was used to measure DPPH activity. Briefly, DPPH (25 ml, 400 mM) was mixed with MES buffer in 0 (25 ml of 0.2 M). The pH 6.0 was accustomed with 1N sodium hydroxide and ethanol (25 ml of 20% v/v). Sample extract (0.9 ml) was mixed with DPPH cation solution (2.7 ml) and placed in dark for 20 minutes at room temperature. Decrease in the absorbance was recorded at 520 nm and the results were calculated as mM AAE (milli mole ascorbic acid equivalent per 100 g of dry weight)

Reducing power (FRAP) assay

Benzie and Strain (1996) assay was employed to measure the ferric reducing antioxidant power (FRAP) of the methanol extract of the samples. FRAP solution was set by mixing 10 vol. of 300 mM acetate buffer (i.e., 3.1 g of NaOAc and 16 ml glacial CH_3COOH per liter), 1 vol. of 10 mM TPTZ (2,4,6-tri-2-pyridyl-1,3,5-triazin) in 40 mM HCl and 1 vol. of 20 mM FeCl_3 . 3 ml of pre-warmed (at 37°C) mixture was mixed to methanolic extract (0.10 ml) and placed at 37°C for 8 minute. The absorbance of the solution was recorded at 593 nm and the results were displayed as mM AAE (milli mole of ascorbic acid equivalent)/ 100 g dry weight.

Antimutagenic activity

Antimutagenic (DNA damage inhibition) property of *P. cirrhifolium* extract was screened using pBR322 plasmid DNA. DNA breakage is regarded as the alteration of the super coiled (S) shape to the open-circular (OC) and/or linear (L) shape (Russo et al. 2011). Each population extracts were screened for DNA damage inhibition activity. Reaction mixture (15 μM) containing 2 μM of pBR322 plasmid DNA (180 ng), 2 μM of 7.5% H_2O_2 , and plant extracts with positive control (+C) and negative control (-C) was prepared. The reaction was conducted at room temperature under UV emission for 20 minutes. Agarose gel electrophoresis was carried out using 0.8% agar in TBE buffer (0.5X) for 2 h at 45 V. The gel was analyzed for presence of fragment and density of the fragments were estimated using Uvi pro Platinum 1.1, gel documentation system (Uvitech, Cambridge,UK). Based on band intensity, band area was calculated and finally converted into percent protection.

DNA isolation

One gram leaf tissue was sliced into sections and grounded into fine powder in liquid nitrogen. DNA extraction was performed using CTAB (cetyl trimethyl ammonium bromide) scheme with slight changes (Jugran et al. 2013). DNA was electrophoresed with 1% agarose gel to verify its purity and presence. The amount of genomic DNA was determined using UV visible spectrophotometer (Hitachi, Japan). The absorbance ratio of DNA sample between 260 and 280 nm was measured and DNA quality was confirmed. The purified DNA sample was stored at 4°C for further analysis.

PCR amplification

A total of 55 RAPD and 45 ISSR primers were initially screened to amplify the DNA samples of *P. cirrhifolium*. Only nine RAPD and ten ISSR markers exhibited clear, reliable and reproducible fragments (Table 2). The PCR reaction was performed using a reaction mixture of 20 μl in a 0.2 ml microfuge tube with 2 μl reaction buffer (10x), 2 μl , 25 mM MgCl_2 , 0.2 μM , each dNTPs, 0.2 μM primer, 20 ng DNA and 1 unit *Taq* polymerase (Genetix, India). The RAPD markers amplification programme was initiated with denaturation of 94°C for 4 minutes 1 cycle, final denaturation at 94°C for 1 minute, primer annealing (37°C) for 2 minutes followed by initial extension (72°C) of 2 minute and 45 cycle and final extension of PCR cycle was kept 72°C for 7 minutes followed by reaction hold at 4°C.

For ISSR, denaturation was conducted for 5 minutes at 95°C, final denaturation for 1 minute at 94°C, primer annealing (46.5-55°C) for 1 minute followed by initial extension (72°C) of 2 minute and final extension of PCR cycle was kept for 7 minutes at 72°C followed by reaction hold at 4°C. The amplified DNA fragments were separated using 1kb DNA ladder (Genetix, India) on a agarose gel (1.5% (w/v) for RAPD marker and 2% for ISSR marker) using TAE buffer (0.5X) and marked with Etbr (ethidium bromide) (0.5 $\mu\text{g}/\text{ml}$). UVI Pro platinum gel documentation system (Version 11.9, Cambridge, UK) was utilized to visualize the pattern of the gel.

Statistical analysis

Data generated for genetic diversity parameters analysis were scored in binary matrix format and used for statistical evaluation. The occurrence of band was demarcated by 1 and absence by 0. Genetic variability attributes like frequency of the alleles, percent of polymorphic loci (Pp%), average allele number per locus (Na), effective number of alleles (Ne), genetic diversity index (He) and

Shannon Information Index (I), was estimated by using POPGENE program version 1.31 (Yeh et al. 1999). Nei genetic distances (D) between populations was analyzed using Nei (1978) and rate of gene flow using standard method (Wright, 1951). Unrooted phylogenetic trees was constructed using Neighbor-joining methods (Saitou and Nei 1987) using NEIGHBOR component of PHYLIP program version 3.68 (Felsenstein 2006). The bootstrapping at 1000 replicates was applied to measure the statistical connotation of the clusters obtained using SEQBOOT, GENEDIST, NEIGHBOR and CONSENSE programmes (Felsenstein 2006). GenAlex version 6.1 (Peakall and Smouse 2006) was used to investigate inter and intra population genetic variations by analysis of molecular variance (AMOVA).

The admixture level and cluster number among population was measured in combined RAPD and ISSR data by Bayesian assignment tests by means of STRUCTURE software version 2.3.4 (Pritchard et al. 2000). Different burning time length and number of MCMC (Markov chain Monte Carlo) replications were set to establish the appropriate set of both parameters initially. STRUCTURE was placed with a burning time length of 100,000 followed by number of MCMC reps after burning 500,000 replications by employing admixture model through previously assigned population as sampling localities and with the among populations with linked alleles frequency. Number of K was established by conducting a total of ten runs (K=1-10) with 3 interactions initially to decrease extra burden of simulation on computer. The maximum probable K value was predicted by ΔK statistics using program Structure Harvester (Earl 2012) to estimate the utmost stage of changes between all succeeding values of K (Evanno et al. 2005). Concluding evaluation for each K value is conducted using K value from 1 to 5 with 20 interactions.

Data on phytochemicals and antioxidant property is analyzed as an average value $\pm$ standard error (SE) of three separate replicates. Significant variations were estimated among average values of populations using DMRT (Duncan's multiple range test ; $p < 0.05$). Correlation coefficients (r) and coefficients of determination (R²) were calculated through SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). R studio was used to develop a correlogram using CORRPLOT programme.

Results

Total phenol, tannin, flavonol and flavonoids

A significant variation was observed in total phenolics (1.89-3.47 mg GAE/g), tannins (1.41-2.50 mg TAE/g), flavonols (1.30-4.05 mg QE/g) and flavonoids (0.71-4.59 mg QE/g). Phenolic content was recorded higher (3.47 mg GAE/g) in Gagar population compared to other populations (Table 3). Total tannin content was found significantly ($p < 0.05$) higher (2.50 mg TAE/g) in Gagar and lower in Satbunga population (1.51 mg TAE/g).

Total flavonols was recorded significantly ($p < 0.05$) higher (4.05 mg QE/g) in Pandavkholi population and lower in Mukteshwar (1.30 mg QE/g) and Gagar (1.31 mg QE/g) populations. Similarly, total flavonoid was found maximum (4.59 mg QE/g) in Mukteshwar population and minimum (0.71 mg QE/g) in Gagar population.

Antioxidant and DNA damage inhibition properties

Methanol extract of the rhizomes of *P. cirrhifolium* evaluated for antioxidant properties by ABTS (3.26-5.12 mM AAE/100 g DW), DPPH (1.09-1.70 mM AAE/100 g DW) and FRAP (0.58-2.33 mM AAE/100 g DW) showed significant variations (Table 3). Considerably ($p < 0.05$) higher ABTS activity (5.12 mM AAE/100 g DW) found in Mukteshwar and lower in Pandavkholi (3.26 mM AAE/100 g DW). FRAP activity was recorded maximum (2.33 mM AAE/100 g DW) in Thanidar population and minimum (0.85 mM AAE/100 g DW) in Pandavkholi population. DPPH was recorded significantly ($p < 0.05$) higher (1.70 mM AAE/100 g DW) in Thanidar comparing to remaining populations except Mukteshwar (1.65 mM AAE/100 g DW) population.

Antimutagenic activity of *P. cirrhifolium* populations was investigated (Figure 1), which is an initial analysis of anti-mutagenic potential against oxidative stress. Gagar population showed significantly ($p < 0.05$) higher DNA recovery percentage (84.95%) followed by Mukteshwar population (82.92%) where as, lower recovery percentage was found in Satbunga population (67.33%).

Genetic diversity and analysis of molecular variance (AMOVA)

Significantly higher genetic diversity was noted in all studied populations of *P. cirrhifolium* using RAPD and ISSR markers. Out of 33 unambiguous and reproducible bands, 30.40 (mean across population) polymorphic RAPD loci were detected. The amplified fragments ranged from 02 (OPA14) to 06 (OPJ3), with an average of 3.6 fragments per primer (Figure 2a). With an average of 92.12%, the polymorphic loci percent (Pp%) ranged from 87.88% (Thanidar) to 96.97% (Pandavkholi). Nei gene diversity index (He) ranged

from 0.362 (Gagar) to 0.303 (Satbunga) with an average value of 0.339 (Table 4). Shannon information index (I) ranged from 0.46 (Satbunga) to 0.53 (Gagar and Pandavkholi populations).

In case of ISSR markers out of 42 unambiguous and reproducible bands, 40 polymorphic ISSR loci were detected using 10 selected primers. The amplified fragments ranged from 03 (UBC810, UBC880, UBC888) to 07 (UBC840) (Figure 2b). With an average of 95.24%, Pp% ranged from 90.48% (Satbunga) to 97.62% (Mukteshwar, Gagar and Thanidar populations). He ranged from 0.253 (Satbunga) to 0.384 (Gagar) with a mean value of 0.320. I ranged from 0.391 (Satbunga) to 0.562 (Gagar) with a mean value of 0.481 (Table 4). AMOVA using RAPD marker demonstrated that the genetic variability was partitioned as 78% within populations and 22% among populations (Table 5). A value of 0.168 genetic differentiations (GST) between populations was detected. The gene flow (Nm) level measured among populations was 2.472. Genetic diversity was partitioned as 73 percent within populations and 27 percent between populations by AMOVA using ISSR markers (Table 5). A high value of 0.221 genetic differentiations (GST) was detected between populations and gene flow was 1.759.

Cluster analysis and population structure

RAPD markers based pairwise Nei genetic distance) revealed minimum (0.069) genetic distance between Satbunga (2276m, grassy slopes) and Pandavkholi (2521m, rocky and boulder areas) populations and higher (0.187) genetic distance between Satbunga (2276m, grassy slopes) and Mukteshwar (2319m, grassy slopes) populations (Table 6). Nei genetic distance was observed to be lowest (0.113) between Thanidar (1984m, open rocky hill slope) and Mukteshwar (2319m, grassy slope) populations and highest (0.250) between Satbunga (2276m, grassy slope) and Pandavkholi (2521m, rocky and boulder areas) populations using ISSR marker (Table 6). Likewise, neighbour joining method based dendrogram separated all studied populations into 2 major groups (i.e. A and B). Group A includes Pandavkholi population and group B with 03 sub groups includes (i) Thanidar and Gagar populations; (ii) Mukteshwar and (iii) Satbunga population (Figure 3a). On the scatter plot, the principal coordinate analysis (PCA) using molecular data reflected the population's heterogeneous distribution (Figure 3b).

The first two principal components [PC 1 (41.54 percent) and PC 2 (26.84 percent)] explained 68.38 percent of the total variation when combined. The relationship among populations in two components is comparable to the clustering pattern. Similarly, dendrogram depended on ISSR markers data using neighbor joining technique divided the locations into two major groups (i.e. A and B). Group A includes Satbunga and group B includes Pandavkholi, Gagar, Mukteshwar and Thanidar. Group B was further divided into 3 subgroups (i) Pandavkholi and Gagar, (ii) Mukteshwar, (iii) Thanidar (Figure 4a). The PCA, using molecular data, reflected the diverse distribution of the population on the scatter plot (Figure 4b). The contribution of first two principal components [(PC 1(36.44%) and PC 2 (29.72%)] explained together 66.15% of the total variance. The relationship among populations in two components is similar to the clustering pattern. Populations structure of *P. cirrhifolium* relying on binary data obtained using both kind of markers was investigated by Pritchard et al. (2000) method revealed that the log likelihood approximations increased regularly as K increased and start to decrease when K= 4 using both markers (Figure 5a). Mean log likelihood plot is prepared by placing values over 10 runs for K values ranging from 1 to 5. The value of K was optimum at 3 as evaluated by the ΔK statistic STRUCTURE based on utilized markers (Figure 5b &c). It was observed attractive that best possible subgroups number was reasonably lower than the total studied populations using both kind of markers, exhibited extensive amount of gene flow, either presently or historically. At probability threshold (Q) of 0.60 using ISSR markers structure analysis, the most of the genotypes were visibly isolated to a definite cluster. Of which, 16 individuals (17.78%) were allocated to cluster-1 and cluster-2 each. Cluster-1 comprises the individuals, mainly collected from Pandavkholi population while cluster 2 possesses the individual gathered from Gagar population. Cluster 3 consist of 55 individuals (61.11%) including the samples from Thanidar, Satbunga and Mukteshwar populations in majority; whereas only 3 (3.33%) individuals were belongs to the admixed group depending on the threshold of 60%.

Relationship among antioxidant, antimutagenic and genetic diversity characteristics

Phytochemical parameters correlated with genetic diversity using RAPD and ISSR marker revealed a considerable ($p<0.05$) positive correlation (Fig. 6a, 6b). Phenol ($p<0.05$; $r=0.924$) and tannin content ($p<0.05$; $r=0.897$) attributed a noteworthy positive correlation) with nei genetic diversity (He) using ISSR marker. Similarly, antimutagenic activity displayed a considerable positive correlation ($p<0.05$; $r=0.898$) with He using ISSR marker. DPPH demonstrated a strong negative correlation ($p<0.01$; $r= -0.968$) with percent of polymorphism (Pp%) using RAPD marker. No other parameter showed relationship with genetic diversity. However, phenol revealed a

noteworthy positive relationship ($p < 0.05$; $r = -0.959$) with tannin whereas, tannin revealed a remarkable negative correlation ($p < 0.05$; $r = -0.903$) with flavonol. ABTS displayed a considerable positive correlation ($p < 0.05$; $r = 0.885$) with DPPH.

Discussion

Medicinal plants serve as a universal remedy for several ailments because of the potent secondary metabolites such as alkaloids, polyphenolic compounds, terpenes, carotenoids etc. These metabolites help plants to cope up with the adverse effect of oxidative stress generated through environmental variables (Suyal et al. 2019a). Phenolic compounds, tannins, flavonols and flavonoids of Himalayan medicinal herbs has been identified for their antioxidant (Giri et al. 2017), anti-inflammatory and anti-cancer activity (Singh and Patra 2018) etc. Therefore, in present study we have investigated total phenolics, tannin, flavonoid, anti-oxidant and anti-mutagenic activity of *P. cirrhifolium* which might be responsible for its medicinal properties. A significant variation was observed in total phenols (1.89-3.47 mg GAE/g), tannins (1.41-2.50 mg TAE/g), flavonols (1.30-4.05 mg QE/g) and flavonoids (0.71-4.59 mg QE/g). Antioxidant property of rhizomes derived methanolic extract of *P. cirrhifolium* as evaluated by ABTS (3.26-5.12 mM AAE/100 g dw), DPPH (1.09-1.70 mM AAE/100 g dw) and FRAP (0.58-2.33 mM AAE/100 g dw) showed significant variation. These findings were in the agreement of Rawat et al. (2013) who reported tannins (2.24 mg TAE/g) and flavonoids (3.05 mg QE/g) in root extract of *Habenaria edgeworthii*. Similarly Giri et al. (2017) reported similar range of phenol, tannin, flavonol and antioxidant property in *Astavarga* species including *P. cirrhifolium* (2.41 mg/g flavonoid; 3.11 mg/g tannins; 1.85 mg/g flavonol; 4.42 mM AAE/100 g dw ABTS; 1.19 mM AAE/100 g dw FRAP). Likewise, similar range of total tannin (2.34 mg CE/g dw) and FRAP activity (0.27-1.78 mM AAE/100 g dw) was reported in *Polygonatum verticillatum* (Patra and Singh, 2018; Suyal et al., 2019).

The antioxidant property of flavonoid from diverse natural resources in the prevention of cellular DNA from damage through inhibition of ROS is documented (Azqueta and Collins 2016). Inhibition of DNA damage is evaluation to detect UV tolerance as a defense arrangement of high-altitude medicinal plants (Suyal et al. 2019a). Ultra violet radiation produces hydroxyl (OH) radical by peroxidizing H_2O_2 , which can damage the plasmid DNA especially the supercoiled form.

Methanolic extract of diverse rhizome part of *P. cirrhifolium* in different population were studied for DNA prevention activity. The rhizome extract of different population showed significant ($p < 0.05$) DNA recovery percentage which is ranged from 67.33- 84.95%. Therefore, extract of *P. cirrhifolium* and other high value medicinal plants could either check or dawdle the process of DNA damage and thus contribute to eliminate main disorders linked to DNA. Reports at the functionality of few Himalayan herbs to inhibit oxidative pressure due to DNA harm are available (Jugran et al. 2016; Giri et al. 2017; Suyal et al. 2019a). Information pertaining to genetic diversity in *P. cirrhifolium* is currently lacking. However, reports are available on genetic diversity studies in other species of genus *Polygonatum* (Kramp et al. 2009; Chung et al. 2014; Meng et al. 2014; Feng et al. 2020). Recently genetic variations of *Polygonatum verticillatum* populations were evaluated using ISSR markers (Suyal et al. 2021). *P. cirrhifolium* has been categorized as an endangered species in entire Himalayan region due to its limited population size coupled with poor regeneration (Suyal et al. 2019b), continuous harvesting, habitat degradation and grazing in the study area.

However, low variation atb genetic level is expected as a general reported trend in endangered plants with smaller population size (Manners et al. 2013). In present study, higher genetic diversity is reported in *P. cirrhifolium* using RAPD (He=0.339; Pp=92.12%) and ISSR (He=0.320; Pp=95.24%) markers. Likewise, many other threatened and endemic plant species despite of their small population size and restricted distribution, comprises high genetic diversity. For instance, Suyal et al. (2021) reported high (He=0.32; Pp%=85.49%) genetic variations in *P. verticillatum* using ISSR marker. Similarly, Naik et al. (2010) reported higher level (He=0.34; Pp%=92.37) of genetic variations in endangered *Podophyllum hexandrum* using RAPD marker; Tabin et al. (2016) in *Rheum webbianum* (He=0.34; Pp%=87%) using ISSR markers; Chaudhery et al. (2012) in *Hedychium spicatum* (RAPD- He=0.44; Pp= 89.58%). The higher genetic diversity in *P. cirrhifolium* might be due to its population distribution in different geographical conditions. In different geographical conditions the plant species may differ in their content due to diverse environmental variables (altitude, temperature, rainfall, humidity, etc.) and breeding system of the species (Zhang et al. 2020). Understanding the genetic variations inside and among populations is critical for basis of powerful and efficient conservation practices for rare species.

Most of the phenotypic variations, in *P. cirrhifolium* allotted inside population [RAPD- 78%; ISSR- 73%]. These results of within population variations are in line with reported variation for some endangered orchids and other threatened herbs e.g., *Dendrobium officinale* [RAPD- 78.88%; ISSR- 78.84%]; *Fritillaria tubiformis* subsp. *moggridgei* [RAPD-82.91%], *Habenaria edgeworthii* [ISSR- 74%] respectively [Ding et al. 2009; Mucciarelli et al. 2014; Giri et al. 2016]. The higher intra population variability of *P. cirrhifolium* can be elucidated based on its life history traits, particularly due to its breeding system. Breeding system of a plant species was found to

influence the distribution of cistrontic diversity in plant populations. Additionally, factors like habitat disintegration, gene flow, and tiny population size additionally reported to contribute to the present difference (Nybom 2004).

The constant of genetic discrimination (GST) and gene flow (Nm) are two important parameters by which the genetic structure of population are often measured. Low genetic discrimination was recorded using RAPD and ISSR markers in *P.cirrhifolium* [RAPD: GST= 0.168; ISSR: GST= 0.221] although it was comparable to the average coefficients i.e. GST = 0.22 reported for out-crossing species (Nybom 2004) and GST=0.231for monocots (Hamrick and Godt 1990). Similar low genetic differentiation as reported elsewhere (Naik et al. 2010; Tiwari et al. 2015).

Factors like geographical isolation, small population size of target species, habitat fragmentation and genetic drift could be responsible for low genetic differentiation (Tiwari et al. 2015; Suyal et al. 2019b). A relatively high correlation between chemotypic and genetic markers were identified. Giri et al. 2016, observed similar results in *H. edgeworthi*, where ISSR marker revealed a noteworthy correlation with total phenolics [t=3.196; p<0.09]; Jugran et al. (2016) in *Valeriana jatamansi* [ABTS with (DPPH, r=0.482; p<0.05; FRAP, r=0.637; p<0.01)]; and Suyal et al. (2019a) in *P. verticillatum* [ABTS (DPPH, r=0.544; p<0.05)].

Conclusion

This study is the first account on the phytochemical properties and genetic diversity of *P.cirrhifolium*. Populations of *P. cirrhifolium* is restricted to specialized small patches and are threatened in wild due to destructive harvesting. Considering habitat specificity, low plant density, and increasing extraction trends, the species need immediate conservation intervention. Genetic diversity assessment, therefore, is important to define conservation interventions for survival of genetic resources particularly of threatened plants with low population density. The Gagar population, with highest genetic variation, phenolic, tannin and antimutagenic property should be considered by priority as an important reservoir of potentially useful genes as the species shows enormous potential to adapt to altering environmental conditions.

Declarations

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Statements and Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflicts of interest

All authors declare no conflicts of interest in publishing this MS.

Availability of data and material

All data linked with this MS is available within this Manuscript.

Code availability

NA

Author contribution

RS, AKJ, and IDB conceptualized the study. RS and AKJ standardized methodology and conducted the experiments. AKJ performed data analysis. IDB contributed in management of resources and funds. RS and AKJ contributed in Writing - Original Draft of the MS. All authors contributed in Writing - Review & Editing of the manuscript.

Ethics approval

This MS is the authors' own original work, which has not been previously published elsewhere. The paper is not currently being considered for publication elsewhere. The paper reflects the authors' own research and analysis in a truthful and complete manner.

Consent to participate

NA

Consent for publication

All the authors are informed and agreed to submit this manuscript to the GRCE Journal for publication.

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Tables

Table 1

Details of *P.cirrhifolium* samples collected from different geographical location and site characteristics

Location	Geographical parameters			Habitat	Collection number
	Altitude (m asl)	Latitude (°N)	Longitude (°E)		
Thanidar	1984	29°58' 5.88"	80° 36'34.56"	Rocky hill slope	GBP5354
Gagar	2163	29° 26' 2.04"	79°36'5.76"	Moist grassy	GBP 5357
Satbunga	2276	29°26'2.40"	79°36'3.60"	Moist grassy	GBP 5358
Mukteshwar	2319	29°28'30.00"	79°38'43.80"	Open grassy, shady	GBP 5356
Pandavkholi	2521	29°50'2.04"	79°28'14.88"	Rocky/boulder	GBP 5365

Table 2

Details of RAPD and ISSR primers used in the analysis of genetic diversity of 90 genotypes of *P.cirrhifolium*

Marker	Primer name	Sequence (5'-3')	Annealing temperature	No. of fragment amplified
RAPD	OPJ3	TCTCCGCTTG	37 °C	6
	OPA3	AGT CAG CCA C	37 °C	3
	OPA8	GTG ACG TAG G	37 °C	4
	OPA9	GGG TAA CGC C	37 °C	3
	OPA12	TCG GCG ATA G	37 °C	4
	OPA13	CAG CAC CCA C	37 °C	4
	OPA19	CAA ACG TCG G	37 °C	3
	OPA20	GTTGCGATCC	37 °C	4
	OPA14	TCTGTGCTGG	37 °C	2
	UBC840	GAGAGAGAGAGAGAYT	55 °C	7
ISSR	HB12	CACCACCACGC	46.5 °C	5
	HB13	GAGGAGGAGGC	53 °C	5
	UBC860	TGTGTGTGTGTGTGTGRA	55 °C	4
	UBC880	GGAGAGGAGAGGAGA	52 °C	3
	UBC859	TGTGTGTGTGTGTGTGRC	55 °C	4
	17898B	CACACACACACAGT	53 °C	4
	UBC810	GAGAGAGAGAGAGAGAT	55 °C	3
	HB9	GTGTGTGTGTGTGG	46.5 °C	4
	UBC888	BDBCACACACACACA	52 °C	3

B = (C, G, T) (i.e., not A); D = (A, G, T) (i.e., not C); R = (A, G); Y= (C, T)

Table 3

Phytochemical composition, antioxidant and antimutagenic activity in selected population of *P.cirrhifolium*

Population	Phenol	Tannin	Flavonol	Flavonoid	ABTS	FRAP	DPPH	AMA
	mg GAE/g	mg TAE/g	mg QE/g	mg QE/g	mM AAE/100g dw			
Thanidar	1.89±0.05 ^c	1.54±0.02 ^c	3.41±0.02 ^b	1.83±0.01 ^b	4.59±0.08 ^b	2.33±0.01 ^a	1.70±0.02 ^a	72.32±1.55 ^{ab}
Gagar	3.47±0.01 ^a	2.50±0.01 ^a	1.31±0.07 ^d	0.71±0.04 ^d	4.06±0.03 ^c	1.05±0.01 ^c	1.58±0.01 ^b	84.95±7.27 ^a
Satbunga	1.80±0.04 ^c	1.51±0.01 ^c	2.43±0.01 ^c	1.83±0.01 ^b	3.29±0.07 ^d	0.58±0.02 ^e	1.30±0.01 ^c	67.33±1.58 ^b
Mukteshwar	2.80±0.06 ^b	2.34±0.05 ^b	1.30±0.01 ^d	4.59±0.01 ^a	5.12±0.31 ^a	1.18±0.01 ^b	1.65±0.03 ^a	82.92±2.66 ^a
Pandavkholi	2.00±0.13 ^c	1.41±0.01 ^d	4.05±0.02 ^a	0.79±0.01 ^c	3.26±0.13 ^d	0.85±0.01 ^d	1.09±0.01 ^d	79.03±3.50 ^{ab}

ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; FRAP- ferric reducing antioxidant power assay; DPPH-2,2-diphenyl-1-picryl-hydrazyl-hydrate; AMA- anti mutagenic activity; *, **, ***, #

Table 4

Genetic diversity analysis of *P.cirrhiifolium* in studied populations using RAPD and ISSR markers

Marker	Populations	Np	Pp (%)	Na	Ne	I	He
RAPD	Thanidar	29	87.88	1.82±0.09	1.59±0.05	0.51±0.04	0.34±0.03
	Gagar	30	90.91	1.85±0.09	1.64±0.05	0.53±0.04	0.36±0.03
	Satbunga	31	93.94	1.94±0.04	1.50±0.05	0.46±0.04	0.30±0.03
	Mukteshwar	30	90.91	1.91±0.07	1.56±0.06	0.49±0.04	0.33±0.03
	Pandavkholi	32	96.97	1.97±0.03	1.62±0.05	0.53±0.03	0.36±0.02
	Mean	30.4	92.12	1.89±0.03	1.58±0.02	0.50±0.02	0.34±0.01
ISSR	Thanidar	41	97.62	1.98±0.02	1.52±0.05	0.48±0.03	0.31±0.02
	Gagar	41	97.62	1.98±0.05	1.67±0.04	0.56±0.02	0.38±0.02
	Satbunga	38	90.48	1.91±0.06	1.41±0.05	0.39±0.04	0.25±0.03
	Mukteshwar	41	97.62	1.98±0.02	1.61±0.05	0.53±0.03	0.36±0.02
	Pandavkholi	39	92.86	1.93±0.04	1.49±0.05	0.45±0.03	0.30±0.03
	Mean	40	95.24	1.95±0.02	1.54±0.02	0.48±0.01	0.32±0.01

Np - Number of polymorphic loci; Pp% - Percentage of polymorphic loci; Na - Observed number of alleles; Ne - Effective number of alleles; He - Nei's (1973) gene diversity; I -Shannon's Information index

Table 5

Analysis of molecular variance (AMOVA) based on RAPD and ISSR markers

Marker	Source of variation	Degree of freedom (df)	Sum of square (SS)	MS	Variance component	Percentage of total variance
RAPD	Among Population	4	148.510	37.128	1.730	22**
	Within Population	85	528.200	6.214	6.214	78**
	Total	89	676.710		7.944	100
ISSR	Among Population	4	226.733	56.683	2.731	27**
	Within Population	85	639.333	7.522	7.522	73**
	Total	89	866.067		10.253	100

Table 6

Nei's Unbiased Measures of Genetic Identity and Genetic distance using RAPD marker

Marker	Population	Mukteshwar	Pandavkholi	Gagar	Thanidar	Satbunga
RAPD	Mukteshwar	0				
	Pandavkholi	0.146	0			
	Gagar	0.126	0.118	0		
	Thanidar	0.121	0.120	0.078	0	
	Satbunga	0.187	0.069	0.132	0.136	0
ISSR	Mukteshwar	0				
	Pandavkholi	0.165	0			
	Gagar	0.131	0.129	0		
	Thanidar	0.113	0.225	0.15	0	
	Satbunga	0.191	0.250	0.14	0.173	0

Figures

Fig. 1

Figure 1

Antimutagenic activity of *P.cirrhifolium* extract. Lane 1- only pBR322; lane 2- pBR322, H₂O₂ and UV exposure; lane 3,4,5,6 and 7- damage pBR322 treated with extract

Fig. 2

Figure 2

PCR amplification pattern of *P.cirrhifolium* (a) obtained by OPA8 RAPD markers; (b) UBC 840 ISSR marker

Fig. 3

Figure 3

a Unrooted genetic distance tree showing the relationships among five populations of *P. cirrhifolium* using RAPD marker [The tree was constructed by using a neighbor-joining method (Felsenstein, 2006). The numbers at the nodes of branches are the confidence values obtained from 1000 replications]

b Projection of 05 populations (90 individual) of *P. cirrhifolium* through PCA based genetic distance using RAPD marker. 1: Mukteshwar; P2: Pandavkholi; P3: Gagar; P4: Thanidar; P5: Satbunga

Fig. 4

Figure 4

a Unrooted genetic distance tree showing the relationships among five populations of *P. cirrhifolium* using ISSR marker [The tree was constructed by using a neighbor-joining method (Felsenstein, 2006). The numbers at the nodes of branches are the confidence values obtained from 1000 replications]

b Projection of 05 populations (90 individual) of *P. cirrhifolium* through PCA based genetic distance using ISSR marker. 1: Mukteshwar; P2: Pandavkholi; P3: Gagar; P4: Thanidar; P5: Satbunga

Fig.5

Figure 5

STRUCTURE analysis of natural populations of *Polygonatum cirrifolium* using combined inter-simple sequence repeat (ISSR) and Random amplified polymorphic DNA (RAPD) markers. (a) K=3 appeared to be the optimal number of clusters by showing the ΔK at its peak; (b) Estimated genetic structure based on K = 3 using a Bayesian framework implemented in the STRUCTURE programme across 3 subpopulations of 165 individuals

Fig.6

Figure 6

a Correlation analysis between phytochemicals, anti mutagenic activity and genetic diversity attributes analyzed through RAPD marker

b Correlation analysis between phytochemicals, anti mutagenic activity and genetic diversity attributes analyzed through ISSR marker. ABTS- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; FRAP- ferric reducing antioxidant power assay; DPPH-2,2-diphenyl-1-picryl-hydrazyl-hydrate; AMA- anti mutagenic activity, He- nei's genetic diversity ; Pp- percentage polymorphism