MOLECULAR AND PHYTOCHEMICAL CHARACTERIZATION AND OPTIMIZATION OF DORMANCY BREAKING TREATMENTS IN *CAPPARIS SPINOSA* L. FROM THE TRANS HIMALAYAN REGION OF LADAKH, INDIA

Thesis Submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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MARCH 2012

DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Molecular and phytochemical characterization and optimization of dormancy breaking treatments in *Capparis spinosa* L. from the Trans Himalayan region of Ladakh, India"** submitted at **Jaypee University of Information Technology, Vaknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Gyan Prakash Mishra** and **Dr. Pradeep Kumar Naik.** I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Molecular and phytochemical characterization and optimization of dormancy breaking treatments in *Capparis spinosa* L. from the Trans Himalayan region of Ladakh, India."** submitted by **Mr. Manish S. Bhoyar** at **Jaypee University of Information Technology, Vaknaghat, India** is a bonafide record of his original work carried out under our supervision. This work has not submitted elsewhere for any degree or diploma.

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ACKNOWLEDGEMENT

The great desire to acquire higher qualifications and pursue the research drove me to promise my parents, teachers, relatives and my friends, a useful research work. My promise was to live up to their expectations, and I kept been my word. I thank my parents for instilling in me confidence and a drive for pursuing my PhD.

I am highly obliged to my esteemed supervisor, Dr. Gyan Prakash Mishra, Scientist, DIHAR and Dr. Pradeep Kumar Naik, Associate professor, JUIT, I extend my deepest sense of gratitude for their encouragement, unfailing support, cogent criticism and constructive thoughts. Their strong research insight made me avid and enthusiastic to do this work with vigor and interest. I could not have imagined having a better advisor and mentor for my PhD study. I deem it a privilege to be a scholar doing research under.

I owe a word of gratitude to Dr. R. B. Srivastava, Director, DIHAR, DRDO, C/o 56 APO, Leh-Ladakh, and Dr. Shashi Bala Singh, Director DIPAS, Delhi (formal Director, DIHAR) for providing obligatory facilities to carry out the research work.

I am indebted to Dr. Ravi Prakash (Vice Chancellor, JUIT), Dr. Y. Medury (CCO, JUIT), Brig. (Retd.) Balbir Singh (Registrar, JUIT) and Prof. R. S. Chauhan (HOD, BT & BI Department). They have throughout helped me in providing all kinds of scientific and administrative assistance to carry out my research work. Present research work would not have been possible without the assistance and active support of the administrative and teaching staff of JUIT. I feel thankful to Dr. Harvinder Singh, Dr. Anil Kant Thakur, Dipankar bhai and Jatin for their critical guidance and kind co-operation.

I feel indebted to Dr. Raghwendra Singh (Senior Scientist, Directorate of Weed Science Research, Jabalpur.) and Dr. Sunil Jadhav (Scientist, DRDE, Gwalior). Their vast knowledge, constant attention, inspiration, helpful suggestions, lively discussions, constructive criticism and confidence in me were of great help in completion of this thesis work.

I cherish the years spent in DIHAR. This research work would not have been possible without the guidance and active support of the scientific and administrative staff of DIHAR. Therefore, in so many ways, I feel indebted to Dr. Basant Ballabh, Sh. Swami saheb, Sh. Targais for their assistance and valuable contributions. I also thank to Sh. Mohd. Bakir (Haji baba) and labor staff of ARU Division for their priceless assistance.

I am very fortunate to have wonderful colleagues Sunil sir, Jitendra, Girish, Gurucharan, Amol, Prabodh, Ashish, Priyanko, Vijay, Prabhat, Jatinder, Richa, Saroj and friends Pawan, Ram, Prashant sir, Vinod sir, Sunil, Amit, Kailash who have always been a constant source of inspiration and helped me in a numerous ways. They have throughout helped me in providing all kinds of assistance to carry out my research work.

I shall remain ever grateful to my school and college teachers specially Prof. A. D. Choudhary, Dr. Nitin Dongarwar, Dr. D.P. Gogle as well as seniors Dr. Rupesh Badere, Dr. Sandeep Hate, Dr. Deepti Saoji, Dr. Walay Tagade, Dr. Mahesh Kawale, Dr. Bhupesh Mendhe, Mr. Shailesh bansod, Dr. Shailendra Gurav, Dr. Manisha Das who has always been inspirational for putting in hard work.

Finally, I extend my deepest sense of gratitude to my Aai and Baba who believed in me and whose support, love, endurance and blessing made me complete this thesis with eager and sincerity. My indebtedness extends to Ashoo, Tai and all relatives for their affectionate motivation, devotion to support me during a specially demanding period of life. Lastly, I extend my apologies to anyone I failed to mention.

(Manish S. Bhoyar)

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LIST OF ABBREVIATIONS

ABTS	:	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
AFLP	:	Amplified Fragment Length Polymorphism
AMOVA	:	Analysis of Molecular Variance
ANOVA	:	Analysis of Variance
AOA	:	Antioxidant Activity
BHT	:	Butylated hydroxytoluene
CC	:	Correlation Coefficient
CRBD	:	Completely Randomized Block Design
CTAB	:	Cetyletrimethyle Ammonium Bromide
DI	:	Diversity Index
DMRT	:	Duncans' Multiple Range Test
DNA	:	Deoxyribose Nucleic Acid
DPPH	:	2,2-Diphenyl-2-picrylhydrazyl
DW	:	Distilled Water
EDTA	:	Ethylenediamine tetraacetic acid
EMR	:	Effective Multiplex Ratio
FRAP	:	Ferric Reducing Antioxidant Power assays
Fst	:	Wright's inbreeding coefficient
GA_3	:	Gibbrelic Acid
GAE	:	Gallic Acid Equivalent
Gst	:	Genetic diversity
H	:	Nei's gene diversity
H_2SO_4	:	Sulfuric Acid
HCl	:	Hydrochloric Acid
HNO_3	:	Nitric Acid
Ht	:	Heterogeneity
I	:	Shannon's Information index
IC_{50}	:	Inhibition coefficient
IDT	:	Integrated DNA Technology
ISSR	:	Inter Simple Sequence Repeats
ISTA	:	International Seed Testing Association
KNO_3	:	Potassium Nitrate
KR	:	Kilo Rads
MQ	:	Milli Q
MSL	:	Mean Sea Level
NJ	:	Neighbor Joining
NPL	:	No. of Polymorphic Loci
PCR	:	Polymerase Chain Reaction
PPL	:	Percent Polymorphic Loci
PVP	:	Polyvinylpyrrolidone

RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribose Nucleic Acid
SPSS	:	Statistical Package for the Social Sciences
Taq Polymerase	:	*Thermus aquaticus*
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
TPTZ	:	2,4,6-tripyridyl-*s*-triazine
USDA	:	United State department of Agriculture
WHO	:	World Health Organization

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ABSTRACT

Capparis spinosa L. (Capparidaceae) also called 'Caper' and locally known as '*Kabra'* is one of the well known medicinal plant in '*Amchi system*' (local medicinal system). It has long been used by the natives of Ladakh as a leafy vegetable and forage. In India, it is found in inner valleys of trans–Himalaya between 3020–3790 m which includes Indus, Nubra and Suru valleys of Ladakh region. It grows in roadside, dry rocky slopes and stony soils, can withstand extreme temperature (–30 °C to +35 °C) of Ladakh and is highly drought tolerant. This plant has multiple uses in cuisine as salad, pickle and condiments. Bio-chemical studies have reported the presence of alkaloids, lipids, flavonoids and glucosinolates, cancer preventing agents and biopesticides in *Capparis spinosa*. In Ladakh, several types of medicinal preparations from *Capparis* are being used by the Amchis (local traditional doctors) for the treatment of various ailments like gastrointestinal infection, diarrhoea and rheumatism. In Ayurveda it is used in splenic, renal and hepatic complaints; root bark is used as tonic, expectorant, anthelmintic, emmenegogue and analgesic. From ancient times, the floral buttons of *C. spinosa* (capers) were employed as flavouring in cooking and are also used in traditional medicine for their diuretic, antihypertensive, poultice and tonic properties. Besides, it brings many environmental benefits, including soil and water conservation, desertification control and land reclamation in fragile cold ecosystem of Ladakh.

Earlier, caper leaves were used only in small quantities by local people for vegetable and roots for crude drug purpose. However, recently this plant is being used more commonly because of its usefulness in food, pharmaceutical and cosmetic industries. Heavy indiscriminate harvesting from the wild along with heavy grazing at high altitude pasture in the trans-Himalayan region of Ladakh has now threatened its survival. Some of the populations in certain pockets have virtually disappeared owing to anthropogenic activities and overexploitation. As the species is already exploited for its aerial and an underground part continues to exceed the rate of natural regeneration, it needs immediate attention for conservation. Caper plants can be propagated through seeds or stem cuttings; however, both methods present serious problems and restrictions to the commercial expansion of this crop. Although capers are widely grown on dry and

poor soil where environmental conditions are not easy for the cultivation of other crops, it is difficult to propagate seedlings because caper seed have both physical (seed coat) and physiological dormancy and it is difficult to germinate naturally. As the dormancy in this crop is due to the hard seed coat, external treatments are necessary to overcome the prevailing dormancy.

Very poor seed germination is the major problem in the wide scale cultivation of capers in the trans-Himalayan region. The present study was conducted to examine the role of various dormancy breaking treatments *viz*. soaking in the warm water, scarification, stratification, conc. acids (H_2SO_4, HNO_3, and HCl), hormone (GA_3), potassium nitrate, alcohol, acetone, gamma ray irradiation and seed soaking in the cow urine on the germination of various caper seeds which was collected from wild plants growing in Ladakh. The seeds were left out in the germination chambers from 20 to 25 days after its treatments. Highest germination rate of 62% was observed when seeds were treated with H_2SO_4 for 40 min followed by 400 ppm of GA_3 soaking for 2 hrs. There are possibilities that hard seed coat was not the only hurdle to the germination and the dormancy was partly due to the physiological restrictions as well. It is recommended to germinate the caper seeds in the petridishes under specified treatments and then transfer these seedlings first in the greenhouse for 2-3 months and then to the open field conditions during spring-summer season (May-August) of Ladakh. The standardization of germination technique will ultimately help in the widespread cultivation of this wonder plant at the farmer's field of Ladakh.

Studies of its population biology and genetic diversity are important for successful development of conservation strategies. An antioxidant activity and polyphenol content varies greatly among populations of *C. spinosa* from the trans Himalayas. Therefore, it would be inefficient, environmentally destructive and economically unsound to randomly harvest *Capparis*. For the rapid development of *C. spinosa* as an economically viable alternative crop, it would be essential to evaluate the natural populations of *C. spinosa* for high antioxidant content and agronomic traits in the trans Himalayan region. Appropriate methods of conservation management should be

adopted including *in situ* conservation and germplasm collection from the remaining populations with great genetic variations.

In order to facilitate reasoned scientific decisions on its conservation management and selective breeding programme. Genetic characterization of 90 wild grown *C. spinosa* individuals were collected from nine sampling sites from three valleys *viz.* Suru (10,756 ft), Nubra (9,600 ft) and Indus (11,500 ft) of trans-Himalayan region, was performed using 40 PCR markers (20 RAPDs and 20 ISSRs). RAPD analysis yielded 223 fragments, of which 220 (98.65%) were polymorphic while, ISSR produced 75 bands, of which all are found polymorphic, with an average of 11.0 and 10.62 polymorphic fragments per primer respectively. ISSR markers were found more efficient with regards to polymorphism detection, as H, I, Ht, Hs, Fst, diversity index and marker index were found more for ISSR than for RAPD marker. The genotypes collected from different locations and valleys were clustered more likely as per their populations based on RAPD, ISSR and in combination of RAPD and ISSR markers. AMOVA analysis showed that total variation aomong genotyes was maximum, followed by among population and least for among valley in all the three cases. Highest occurrence of molecular variation among genotypes revealed that it is require conserving maximum number of genotypes from different valleys of trans-Himalayas to protect the gene pools and for future breeding program.

For conservation aspects it is very important to assess the potential of *C. spinosa* populations for high antioxidant activity and phenolic compounds. Antioxidant activity (%) of *C. spinosa* leaves collected from nine different sites from the Ladakh region were measured using three different methods i.e. 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) activity, 2,2-azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity and ferric reducing/antioxidant power assay (FRAP). Total phenolics were measured using Folin-Ciocalteau method and total flavonoid content using spectrophotometric method in the methanolic extract of *Capparis* leaves. Maximum DPPH and ABTS radical scavenging activity was observed from Skuru and least from Tirchey samples. FRAP assay revealed that Skuru samples possess maximum

antioxidant content as compared to the samples from any other location. The IC_{50} value of ABTS were quite reasonably correlated with FRAP assay (R^2=0.517). However, IC_{50} of DPPH was found poorly correlated with both ABTS (R^2=0.100) and FRAP assay (R^2=0.223). The highest phenolic and flavonoid content was found in Skuru samples and lowest in Tirchey samples. The total phenolic content (27.62-21.42 mg GAE/g DW) and total flavonoid content (6.96-2.69 mg quercetin equivalent/g DW) were reasonably correlated with IC_{50} value of ABTS (R^2=0.741 and 0.703 respectively) and FRAP (R^2=0.605 and 0.649 respectively) but poorly correlated with DPPH IC_{50} value (R^2=0.303 and 0.407 respectively). The result of the present study confirm antioxidant potential of the leaves of *C. spinosa* collected from different locations of Ladakh region, whose phyto-chemistry and phyto-pharmacology should be investigated further in order to detect possible phyto-therapeutic uses where free radicals are implicated.

The antioxidant activity and total polyphenols of the methanolic extract of all edible parts such as leaves, flower buds, roots and fruits of *C. spinosa* were assessed in an effort to corroborate the medicinal and culinary potential of the edible parts of the plant. To estimate the mentioned antioxidant capacity, three different methods were performed: the 2,2-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity and ferric reducing/antioxidant power assay (FRAP). To evaluate the total phenolic contents, the assay using Folin-Ciocalteu reagent was used and total flavonoids were estimate by spectrophotometric method. Highest DPPH and ABTS radical scavenging activity was observed in leaves and least in dried fruit samples. FRAP assay illustrated that leaves samples possess maximum antioxidant contents and dried fruit sample restrain minimum as compared to the other edible parts and well-known antioxidant butylated hydroxytoluene (BHT). The IC_{50} value of DPPH were highly correlated with IC_{50} value of ABTS (R^2=0.9074) and FRAP assay (R^2=0.9771). However, IC_{50} value of ABTS reasonably correlated with FRAP assay (R^2=0.5737). The highest phenolic and flavonoid content was found in leaves samples (24.77-5.69 mg GAE/g DW) and lowest in dried fruit samples (4.07-0.00 mg quercetin equivalent/g DW). The total phenolic contents were highly correlated with IC_{50} value of ABTS (R^2=0.9074), DPPH (R^2=0.9377) and

FRAP value (R^2=0.9617). But total flavonoid contents were reasonably correlated with ABTS (R2=0.7449), DPPH (R^2=0.7791) and FRAP value (R^2=0.9577). This study, has to some extent, validated the medicinal potential of all the edible parts of the *C. spinosa*.

Pickle of capers has high demand in European countries as it has tremendous antioxidant potential, hence physico-chemical properties of flower buds collected from Ladakh region were measured using different assays. Nutritional compositions including carbohydrates, protein, ash, moisture content, minerals and vitamins was compared with reported value. Caper buds collected from the Ladakh region were found to be a better source of protein and minerals as contrast to the reported compositions. It can supplement the calorie requirement of local people and the army deployed in the 'Ladakh' sector.

CHAPTER 1

INTRODUCTION

For centuries, traditional societies have exploited edible wild plant resource to obtain their nutritional requirements [1]. There has been renewed or increasing interest in consuming wild food plants [2, 3]. Millions of people in many developing countries do not have enough food to meet their daily requirements and a further more people are deficient in one or more micronutrients [4]. Despite agricultural societies' primary reliance on crop plants, the tradition of eating wild plants has not completely disappeared, their nutritional role and health benefits being reported in many surveys worldwide [5, 6]. The diversity in wild species offers variety in family diet and contributes to household food security. A study conducted in Zimbabwe revealed that some poor households rely on wild fruits as an alternative to cultivated food for a quarter of all dry season's meals [7]. Similarly, in Northern Nigeria, leafy vegetables and other bush foods are collected as daily supplements to relishes and soups [8]. In Swaziland, wild plants is still of great importance and contribute a greater share to the annual diet than domesticated crops. Various reports also noted that many wild edibles are nutritionally rich and can supplement nutritional requirements, especially vitamins and micronutrients [9, 10]. Nowadays, however, consumption is determined less by calorie input and more by the pleasure of gathering wild resources, recreating traditional practices and enjoying characteristic flavors [11, 12]. In addition, wild plants are valuable source of a wide range of secondary metabolites which are used as pharmaceuticals, agrochemicals, flavor, fragrance, color, biopesticides, food additives and many more. Over 70 percent of the approximately 30,000 known natural products are of plant origin [13]. Worldwide, 121 clinically useful prescription drugs are derived from plants [14].

The World Health Organization (WHO) also estimated that approximately 70 percent of the world populations rely primarily on traditional medicine as source for their primary health care [15]. More recent statistics based on prescription data from 1993 in the United State reveals that over 50 percent of the most prescribed drugs had a natural product either as the drug or as the starting point in the synthesis or design of the actual end chemical substances [16]. Domestication and cultivation of wild edible plants is,

therefore, essential in broadening the food base in developing countries. This will lead to diversification, which will ensure a dietary balance and intake of micronutrients.

Ladakh is a part of Indian Himalaya at an altitude of 3020-3790 m above mean sea level, is characterized by diverse and complex land formations. It has many unconquered peaks of impregnable heights, uncharted glaciers and valleys. It is located at the latitude of 31º 44' 57" – 32º 59' 57" N and longitude of 76º 46' 29"– 77º 41' 34" E which covers more than 65,000 sq km area and is characterized by low annual precipitation (20-30 mm rainfall/snowfall), temperature ranges between +35º C in summer to -35º C during winter along with low relative humidity (20-40 %). These climatic features make this region a typical cold arid dessert. Under these unique geographical position and adverse climatic conditions many plant species were able to establish themselves and majority of such plants were nutritionally as well as medicinally potential and suitable to use as non-conventional vegetables. These plants were identified by local people through over the years of experience perhaps via trial and error method which led to the selection of plants that are edible. These selected plants were gradually added to the repertoires of the edible plants [17].

Due to lack of proper records and over-exploitation of these wild edible plants by local people; the natural resources along with related indigenous knowledge are depleting day by day [18, 19]. It is worthwhile to note that the amalgamation or maintenance of edible wild plants resources could be beneficial to fulfill the total vegetable requirement of the locals as well as the army deployed in these harsh regions of the country mainly due to its high perishable nature and involvement of long distance transportation especially during winters when the region remains land locked due to heavy deposition of snow on the road passes. Air lifting of the fresh vegetables results in heavy expenditure which will not be affordable to the people. Therefore, there is need to create social awareness among local tribal communities about ecological importance, cultivation and sustainable harvesting of non-conventional wild high altitude plants which have potential for culinary as well as medicinal uses.

In India, very little is known about *C. spinosa* cultivation techniques, its utilization, genetic variation and the potential crop improvement through domestication, selection or breeding under Ladakh condition. The urgent need is standardization of seed germination as well as agronomical practices, genetic diversity study for future breeding programme and proper phytochemical investigation as well as nutritional profiling of selected plant species like *C. spinosa* pertaining to its pharmacological importance as well as for its use as food supplements. These plants could be included in the list of our agricultural/ horticultural crops as a new potential source of food as well as medicinal plants after proper analysis. The caper bush (*Capparis spinosa*) is one such wild vegetable, which grows as an underutilized wild edible plant in Ladakh region, but is a semi-cultivated popular leafy vegetable in many parts of European countries.

1.1 Research Plant Profile

Capparis spinosa (Capparidaceae) (Figure 1.1) also called 'Caper' and locally known as '*Kabra'* is one of the oldest known medicinal plant of Amchi system (Local doctors) which is occasionally used by local people of Ladakh as a leafy vegetable and forage. It is an underutilized wild plant which grows in roadside, on the slopes, dry, rocky and stony soils, can withstand extreme temperature (-40 °C to +40 °C) of Ladakh and is highly drought tolerant. This plant has multiple uses in cuisine as salad, leafy vegetable, pickle and condiments. Besides, it also possesses many environmental benefits, including soil and water conservation, desertification control and land reclamation in fragile cold ecosystem of Ladakh due to its canopy coverage and extensive root system [20].

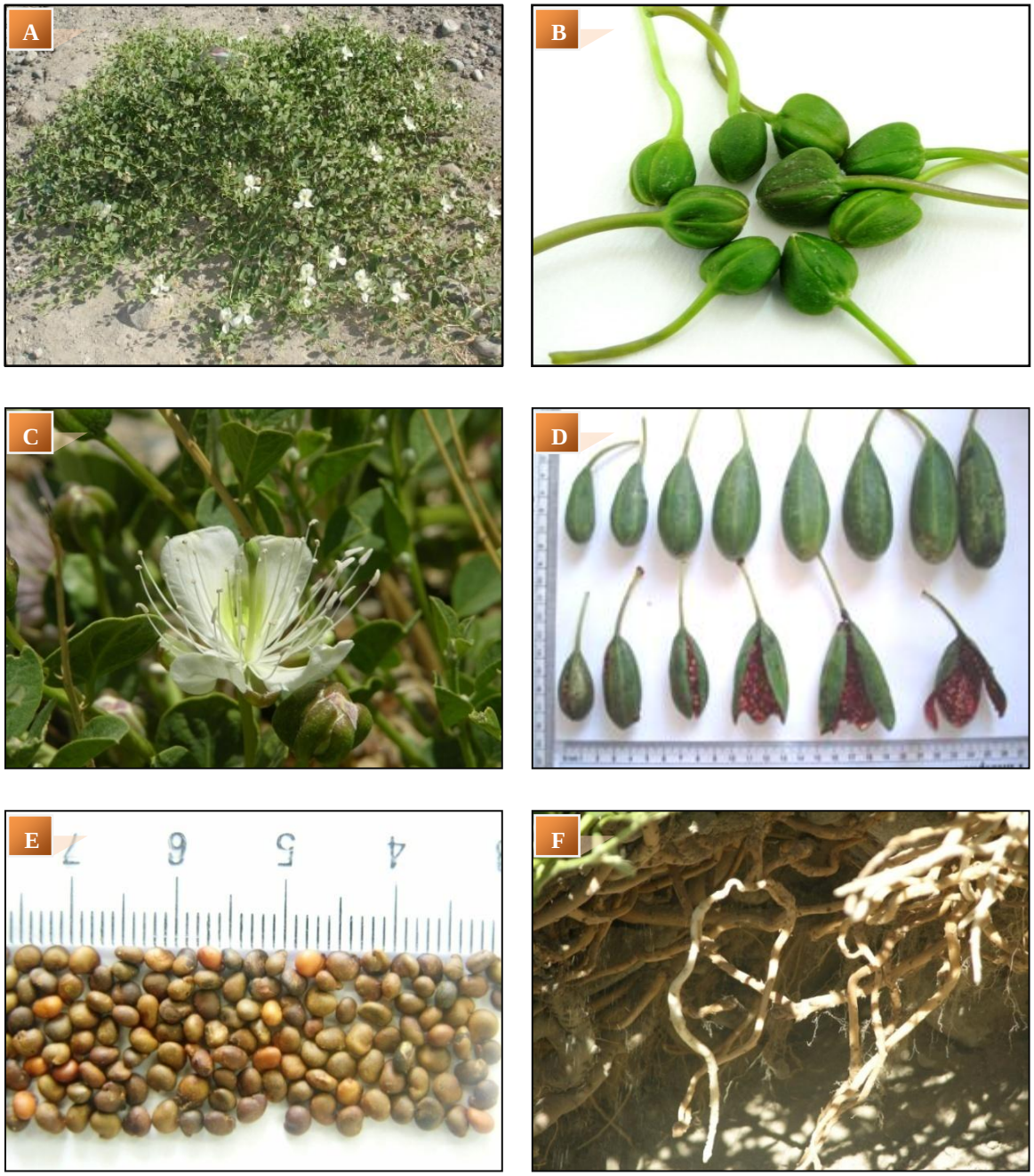


Figure 1.1 The plant *Capparis spinosa* and its different parts. **A.** Habitat **B.** Flowering plant at natural habitat **C.** Flower buds **D-E.** Flowers **F.** Fruits **G.** Seeds **H.** Root system

It is highly nutritious plant having immense medicinal properties and is used for treating multiple diseases. It can also be consumed as salad, pickle and condiments. It can meet the calorie requirement of local people and has immense potential as the leafy

vegetable for the troops deployed in the Ladakh sector especially during winters when roads are closed. The plant has immense potential outside the region, both as vegetable and medicinal plant [21]. Previous chemical studies on *C. spinosa* from other parts of the world have reported the presence of alkaloids, lipids, flavonoides and glucosinolates, which are naturally occurring product belonging to the order capparales, known as flavour compound, cancer preventing agent and biopesticide [22]. Methanolic extract from *Capparis spinosa* buds exhibited good antioxidant activity [23]. This brings attention to the antioxidant potential of an extract of *Capparis* flower buds that are widely used as a source of flavour. Further it has immense medicinal properties. The root bark is analgesic, anthelmintic, antihaemorrhoidal, aperients, deobstuent, depurative, diuretic, expectorant, tonic and vasoconstructive [24]. The leaves are bruised and applied as a poultice in the treatment of gout. The leaves of *Capparis* are also been used for treating earache, coughs, expelling stomach worm, and for diabetes [25].

Capparis spinosa in the Ladakh region cover almost 10 to 15 m^2 in a place and thus being very important for protecting soil erosion, water conservation, desertification control and land reclamation in fragile cold ecosystem. Furthermore, it also helps in improving the micro-climate of soils. For an example four bacterial strains, *Pseudomonas stutzeri* var. mendocina, *Comamonas sp.*, *Agrobacterium tumefaciens* bivar. 2 and *Sphignobacterium sp*., isolated from the rhizosphere of wild grown caper (*Capparis spinosa* L.) plants were able to fix N_2 as shown by their growth in nitrogen-free medium and by the acetylene reduction test [20].

1.1.1 Botany and Taxonomy of *Capparis spinosa*

Caper bush (*Capparis spinosa* L.) belongs to the botanical family Capparaceae (formerly Capparidaceae). The family Capparaceae comprises 39 genera and 650 species distributed through warm region [26]. Capparaceae are well suited for systematic and evolutionary inquiries. It has been almost universally agreed that Capparaceae have a very close phylogenetic relationship with Brassicaceae, including the model species *Arabidopsis thaliana* [27]. The Genus *Capparis* Comprises 250 species including shrub,

trees, and woody climbers [28]. The chromosome number (2n) of *Capparis spinosa* is 37 [29]. The main germplasm collections are located in Italy and Spain [30]. The genus *Capparis* is classified according to Benthum & Hooker's system of classification as follows:

Division:	Anthophyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Capparidales
Family:	Capparaceae
Genus:	*Capparis*
Species:	*spinosa* L.

Capparis spinosa is a winter-deciduous, diffuse, prostrate, glabrescent, perennial shrub or climber armed with divaricated light yellow thorn**.** Leaves are alternate, variable in texture, round to ovate, orbicular to elliptic, base rounded with mucronate apex. Leaf stipule may be formed in to spine, this is the reason it is called *spinosa*. Flower white to purple, solitary axillary. Flowers are about 2 inches in diameter, white with numerous stamens, and very pleasing in appearance. Reputed to be quite fragrant, the flowers open at dawn and close by late afternoon, during which time they are magnet for insect pollinators. *Capparis spinosa* previously believed to be hermaphroditic [31, 32]. However, in preliminary study in the desert regions of the northern part of Xinjiang, China, found that it is perfect as well as andromonoecious [33]. Fruits are fleshy, oblong ellipsoid with red flesh and many brown seeds. Seeds are kidney shaped and brow to black in colour which is 3-4 mm in diameter. Mature fruits, are dehiscent. Plants have been reported with 6-10 m long roots. The root system may account for 65% of the total biomass [34]. Caper canopy is made up of 4-6 radial decumbent branches from which many secondary stem grow.

1.1.2 Other important species of *Capparis*

According to George H. M. Lawerence [35] the pantropical genus *Capparis* includes 350 species; including the following:

Capparis brevispina - Indian caper

Capparis deciduas (*Capparis aphylla*) - (India, Arabia, North Africa)

Capparis cynophallophora (*C. jamaicensis*) Jamaica Caper Tree

Capparis flexuosa L. - Bay Leaf Caper

Capparis horrida (syn. *C. zeylanica*) (Tropical Asia and Malaysia)

Capparis mariana - formerly grown as a commercial crop in Guam

Capparis micrantha - Melada, Caper Thorn. SE asia, Indonesia

Capparis michellii - Aboriginal Pomegranate, Wild Orange (Australia)

Capparis montana (Aublet) Lemee (syn. *Voyara montana*) Tree from French Guiana

Capparis mooni - from India

Capparis nobillis - Wild Lime (Australia)

Capparis ovata - Caper (Mediterranean)

C. pittieri edible fruit (Tropical America)

Capparis umbonata - native to Australia

Capparis sepiaria - Indian Caper (Asia and East African Coasts)

Capparis tomentosa – Kowangee, Cooked leaves are eaten in times of famine (Tropical Africa)

1.1.3 Ecology and distribution of *C. spinosa*

There is a strong association between the caper bush and oceans/seas. *Capparis spinosa* is said to be native to the Mediterranean basin, but its range stretches from the Atlantic coast of the Canory Island and Morocco to the Black Sea of the Crimea and Armenia, and Eastward to the Caspian Sea in to Iran. In India, it is found in the inner valleys of Himalayas between 3020-3790 m which include Indus, Nubra, and Suru valleys of Ladakh region (Figure 1.2) [36].

In Ladakh region it grows as wild especially around 11,000 ft above mean sea level. The plant can withstand extreme temperature from -40 °C to +40 °C and is drought tolerant. Dry heat and intense sunlight make the preferred environment for caper plants. *Capparis* plant is hardy and grows in roadside, on the slopes, dry, rocky and stony soil.

These characteristics can be explored as a source of important genes for developing abiotic stress tolerant plants. Caper plants grow well in nutrient poor sharply-drained gravelly soils. Mature plants develop large extensive root systems that penetrate deeply into the soil [36].

Figure 1.2 The plant *Capparis spinosa* growing naturally under Ladakh conditions.

Capers are salt-tolerant and flourish along shores within sea-spray zones. Caper plants are small shrubs, and may reach about one meter upright. However, uncultivated caper plants are more often seen hanging, draped and sprawling as they scramble over soil and rocks. The caper's vegetative canopy covers a soil surface which helps to conserve soil water reserves. Leaf stipules may be formed into spines. Flowers are born on first-year branches [36].

1.2 Seed germination of *Capparis spinosa*

Caper plants can be propagated through seeds or stem cuttings; however, both methods present serious problems and restrictions to the commercial expansion of this crop. Although capers are widely grown on dry and poor soil where environmental conditions are not easy for the cultivation of other crops, it is difficult to propagate seedlings because caper seed have both physical (seed coat) and physiological dormancy and it is difficult to germinate. Fresh caper seeds germinate readily, but in low percentages (1–2 %), whereas drying of seeds induces severe dormancy, which is difficult to overcome naturally [37].

Commercial propagation of capers is complicated by limited and variable seed germination under natural conditions of Ladakh. One of the main problems that prevent sustainable use of the plant under Ladakh is that both under laboratory or natural conditions the plant shows highly variable germination, due to seed dormancy. The problem becomes more serious since its plantations are required for commercial production in a sustainable manner. High germination percentage is a must to ensure high plantation and viability. As the dormancy in this crop is due to the hard seed coat, external treatments are necessary to overcome the prevailing dormancy. The structure of the seed and the mucilage which develops when the seed is placed in contact with water could impose an effective barrier against the diffusion of oxygen to the embryo [38]. Previously published reports worldwide related to seed germination practices and breaking seed dormancy of *Capparis* is summarized in Table 1.1

Table 1.1 Seed germination study of *Capparis sp.*

Species	Germplasm collected	Achievements	Reference
C. spinosa	Argentina	Highest germination percentage were obtained using conc. H_2SO_4, followed by either a 90-min soaking procedure in a 100 ppm gibberellins solution or adding 0.2% potassium nitrate to the substrate.	Sozzi et al. [39]

C. ovata Desf. Var. *Palaestina* Zoh.	Turkey	The maximum germination percentage (55%) was obtained in the seeds scarified by P320A sandpaper thickness with GA3 solutions of 400 ppm for 2 hour.	Toncer et al. [38]
C. ovata Desf.	Turkey	A germination percentage of 27.4% was obtained in seeds which were soaked 300 mg L^{-1} GA_3 for 3h after treatment with H_2SO_4 for 30 min. and a germination percentage of 49.7% was provided by soaking seeds in 0.2% KNO_3 for 7 h after treatment with H_2SO_4 for 20 min.	Olmez et al. [37]
C. ovata Desf. Var. *Palaestina* Zoh.	Turkey	The highest seed germination was recorded from scarified 5.77 mM GA_3 treated seeds at an alternatively changing temperature under a photoperiod of 12 h light and 12 h dark.	Soyler et al. [40]
C. ovata Var. *Herbacea*	Turkey	The highest seed germination rate of 61% was obtained from the seeds treated with GA_3 for 24 h.	Soyler et al. [41]
C. ovata	Turkey	The experiment revealed that the different temperature treatments were effective on mean germination obtained at control seeds with 7.39% dry heat treatments effected germination rate, but it was not enough for removing germination obstacle of caper seeds completely.	Basbag et al. [42]
C. spinosa var. *parviflora*	Iran	The highest seed germination (60%) and time to germination (17.2 days for 50% germination) were obtained under sulfuric acid application for 30 min. followed by 200 to 400 mg $^{L-1}$ of GA soaking.	Bahrani et al. [43]
C. spinosa L.	B & T World Seed Co. France	Scarification with 1% H_2SO_4 for 20 min followed by 0.04% GA_3 and one week chilling at 4°C was the most effective in breaking dormancy of dry caper seeds and resulted in 64% germination in commercial substratum containing a mixture of slightly decomposed peat and more strongly decomposed peat with organic matter less	Suleiman et al. [44]

		than 95% and nutrients like N_2 (100-300 mg l^{-1}), phosphate (100-300 mg l^{-1}), and potassium (100-400 mg l^{-1}) as compared to warm water treatment with three months chilling, which gave 42% germination.	
C. spinosa L.	Spain	A soaking period of 30 days or longer enhanced seed germination; final germination values ranged from 95 to 99%, reducing the time to reach 50% of final germination and consequently the duration of germination tests. Addition of gibberellic acid to the substrate after soaking improved germination only for control seeds and those soaked for 24 h or 15 days. Seed soaking for 30 or 45 days, followed or not by the addition of a gibberellic acid solution to the substrate, is an efficient method to enhance caper seed germination.	Pascual et al. [45]

Overall the research works carried out so far on seed germination of caper are scanty and insufficient across the world. Moreover, till date there is no report about caper seed germination from cold arid desert of trans–Himalayan region of Ladakh.

1.3 Genetic diversity of *Capparis spinosa* populations

There is lack of information on the genetic diversity of caper germplasm. Most capers are gathered from natural habitat with little attention to the preservation of germplasm resources and genetic diversity. There is a need to carry out preliminary studies to assess the extent of genetic variation between and within natural population of caper, as well as to assess techniques for sustainable harvesting and further adaptation of domesticated varieties. Some investigation and characterization of capers genetic diversity has been done by using molecular markers, which is presented in Table 1.2

Table 1.2 Genetic diversity assessment of *Capparis sp.*

Species	Germplasm collected from	Tools	Achievements	Reference
C. spinosa L.	Tunisia and Italy	RAPD	Analyzed genetic diversity among 110 genotypes collected from Tunisia and central Italy using RAPD marker.	Khouildi et al. [46]
Capparis sp.	Spain, Morocco and Syria	AFLP	Evaluated phylogenetic relationship among 45 accessions from Spain, Morocco and Syria.	Inocencio et al., [47]
C decidua (forssk.) edgew.	Saudi Arabia	RAPD	Estimated genetic interrelationship among 14 (12 individuals from Raudhat Khuraim and two from the control population) individuals of Raudhat Khuraim and between Raudhat Khuraim and the control population.	Abdel-Mawgood et al. [48]
C. spinosa L.	Italy	RAPD & ISSR	To detect the genetic fidelity of regenerated plants two different DNA-based techniques were used.	Carra et al. [49]
C decidua (forssk.) edgew.	India	RAPD	Genetic variability present in 20 plants from Fatehpur region of Rajasthan was investigated using molecular markers.	Vyas et al. [50]
C decidua (forssk.) edgew.	Saudi Arabia	RAPD	Genetic diversity within and among six populations (Madina, Farasan island, Hawayer Assos, Khor Assos, Raudhat Khuraim, and Taif) of *C. decidua* was studied using RAPD technique.	Abdel-Mawgood et al. [51]

For the purpose of efficient conservation and successful breeding programmes, it will be prudent to study the populations of *C. spinosa* at genetic and molecular levels. Study within and between populations variations at the molecular level provides an efficient tool for taxonomic and evolutionary studies and for devising strategies to protect the genetic diversity of the plant. Genetic variability also can be exploited to select useful genotypes that could be utilized as cultivars to avoid batch-to-batch variations in extraction of standard drugs.

The emphasis of modern agriculture on maximizing productivity has been achieved in part by the replacement of large number of genetically heterogeneous local genotypes or landraces with a small number of genetically uniform but high yielding varieties. Reduction in area under wild habitat due to extension of cultivation, urbanization and environmental degradation has drastically reduced the land available to support viable populations of wild and weedy relatives of crop plants. Thus, crop gene pool diversity comprising both cultivated and wild forms, has been considerably reduced during the last three decades [52]. Without a continuous source of variability, the ability of plant breeders to improve agronomic performance that is based on complex genetic combination could decline [53]. Hence, conservation and proper documentation of genetic resources assumes great importance.

An appropriate use of molecular markers techniques requires to clearly define the issues addressed, what type of information will be needed (on genetic diversity). For accurate and unbiased estimates of genetic diversity adequate attention has to be devoted to: (i) sampling strategies, (ii) utilization of various data sets on the basis of the understanding of their strengths and constraints, (iii) choice of genetic similarity estimates or distance measures, clustering procedures and other multivariate methods in analyses of data; and (iv) objective determination of genetic relationships. For all these reasons, choosing the most appropriate technique may be difficult and often a combination of techniques are required to gather the information [54].

Classical approaches for identification and analysis of genetic variability in crops are based on morphological, physiological and agronomic traits [55]. However, these traits have limitations as they are few in numbers and influenced by environmental factors. Alternatively, the DNA markers are more suitable for study the genetic variability among the populations as these are independent of environmental conditions and are unaffected by developmental stages of the plant. The evaluation of genetic diversity and construction of linkage maps would promote the efficient use of genetic variations in the breeding program [56].

The PCR technology has offered new marker systems for diagnosis of genetic diversity in large scale studies [57]. Over the last 17 years, PCR technology has led to the development of two simple and quick techniques called Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR). These markers have been used both for DNA fingerprinting [58, 59] and population genetic studies [60]. Molecular genetic markers would aid the long-term objective of identifying diverse parental lines to generate segregating populations for tagging important traits, such as gene(s) for high content of rutins, tocopherol etc from these *Capparis spinosa* genotypes.

1.4 Use of molecular markers for studying genetic diversity

In simple terms, genetic diversity is a statistical concept referring to the variation within the individual gene loci among alleles of a gene or gene combinations, between individual plants or between plant populations. The variation on the morphological, production biology and pharmaceutical characters has been attributed to environmental or genetic factors [61]. In recent years, DNA-based molecular markers have been used for assessment of genetic diversity between germplasm in many plant species. DNA-based molecular markers are free from environmental modulations.

They provide increased accuracy and expanded scope of inferring genetic variability within and between populations of plant species. Traditionally, studies of population genetic structure have used proteins, isozymes, allozyme etc. [62, 63]. Only a

little portion of the genome was covered when protein and isozymes were analysed and most part of the genome remained unanalysed. So biochemical characterization does not reflect the entire genome. Furthermore, all genetic changes that occur at the DNA level are not detected at the protein level. The detection of genetic variation is limited to protein coding loci which may not be the representative of the entire genome, hence may lead to underestimation of genetic diversity [64].

Enzymes are tissue and species specific and change during development and differentiation. It is likely to get more variability in DNA than in proteins since much of the DNA does not code for active genes and is unexpressed. Analysis of such variations can be done in the form of DNA profiling. Molecular markers, unlike morphological markers are stable and have been found useful in population studies [65, 66] and phylogeny [67].

1.4.1 Types of Molecular markers

There are various types of DNA markers available presently to evaluate DNA polymorphism in sample genomes. These include restriction fragment length polymorphism (RFLP); simple sequences repeat (SSR), inter simple sequence repeats (ISSR), random amplification of polymorphic DNA (RAPD) [68, 69] and amplified fragment length polymorphism (AFLP) [70]. RFLPs are well suited for the construction of linkage maps because of their high specificity and are also less polymorphic, more expensive and laborious compared to RAPD and ISSR. Selection of a correct marker system depends upon the type of study to be undertaken and whether that marker system would fulfill at least a few of the mentioned characteristics such as easy availability, highly polymorphic nature, Mendelian inheritance, frequent occurrence in genome, selective neutral behavior, easy and fast assay, high reproducibility, free of epistasis and pleiotropy etc. [71].

1.4.2 RAPD Markers and genetic diversity studies

Random amplified polymorphic DNA was introduced by Williams et al. [68]. It is a PCR-based technique that has been applied to study the populations and proved to be very useful tool providing a convenient and rapid assessment of genetic differences between genotypes. Moreover, RAPDs use arbitrary primers that provide a large number of multilocus markers and can be applied to analyze almost any organism, even those for which no previous genetic or molecular information is available. RAPDs are one of the families of techniques that produce arbitrary fragment length polymorphism and are collectively described as multiple arbitrary amplicon profiling [72]. The RAPD technique utilizes single, arbitrary, decamer DNA oligonucleotide primers to amplify regions of the genome using PCR [73, 68, 74]. Priming sites are thought to be randomly distributed throughout the genome and polymorphism in these regions results in different amplification products. The methodology is simple and has been widely used for the assessment of genetic diversity, genetic variation within species, determining relationships between closely related species and genotypes within a species to identify particular genotypes. RAPD technique has also been used to study and investigate clonal and population structure [75]. RAPD Analysis of Maize somaclones was done by Osipova et al. [76].

The use of RAPD for determination of genetic relationships has been demonstrated in a number of crop species like maize [73], Sorghum [77], pigeon pea [78] rapeseed [79], aromatic rice [80] and many medicinal plant species such as *Digitalis obscura* L. [81], *Cymbopogon* species [82], *Echinacea* [83], *Orobanche sp* [84], *Cunila galioides* Benth. [85], *Ocimum basilicum* L. [86], *Lonicera caerulea* [87], *Punica granatum* L. [88] and *Prunus armeniaca* L. [89].

The simplicity of the technique and the speed of data generation have attracted many researchers, particularly those interested in either genetic fingerprinting or the patterns and levels of genetic diversity [90, 91]. In addition to the studies of genetic diversity there have been an increasing number of papers concerned with population

genetics [92] phylogenetics [93] and hybridisation/introgression Durham *et al.* [94] worked on evidence of gene introgression in apple using RAPD markers. Rieseberg [95] suggested that RAPDs may be useful for investigation within species or between closely related species. Many reports are available on inter and intra generic genetic diversity and molecular phylogeny using RAPDs such as, *Arachis* [96], *Pistacia* [97] etc. The usefulness of RAPD fingerprinting was also reported for identification of Italian grape (*Vitis vinifera*) varieties [98] and determining the phylogenetic relationship for 27 tropical maize varieties [99].

All the above studies confirmed the efficiency of RAPD markers for systematic investigations. Thus, it has been suggested that RAPD fingerprinting method is simple and so powerful that one primer can distinguish between different clones while the use of multiple primers reduces fingerprint similarity and resolves discrepancies. RAPD analysis is a very good starting point for studies of relationships within and among closely related species. Only one report was so far available on RAPD characterization of *C. spinosa* collected from Tunisia and Italy [46].

1.4.3 ISSR Markers and genetic diversity studies

Inter Simple Sequence Repeats (ISSR) is a type of molecular marker that can be carried out without prior knowledge of DNA sequence in the genome. Microsatellites (SSR) represent the most abundant source of polymorphism from repetitive sequences. SSR are often used as molecular markers even if this technology is time consuming and expensive. ISSR is an alternative technique to study polymorphism based on the presence of microsatellites throughout the genomes [100]. ISSR markers are DNA sequences delimited by two inverted SSR sequences composed of the same units which are amplified by a single PCR primer, composed of few SSR units with or without anchored end. ISSR-PCR gives multilocus patterns which are very reproducible, abundant and polymorphic in plant genomes [101]. This approach named Inter-SSR (ISSR) employs oligonucleotides based on a simple sequence repeat anchored or not at their 5’- or 3’-end by two to four arbitrarily chosen nucleotides. This triggers site-specific annealing and

initiates PCR amplification of genomic segments which are flanked by inversely oriented and closely spaced repeat sequences.

The marker system called ISSRs has been developed as an anonymous RAPDs-like approach that accesses variation in the numerous microsatellite regions dispersed throughout various genomes (particularly the nuclear genome) and circumvents the challenge of characterizing individual loci that other molecular approaches require [102]. The resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocous marker system useful for fingerprinting, diversity analysis and genome mapping. The first paper on ISSR molecular marker was published by Zietkiewics et al. [100]. In it, Inter simple sequence repeat (ISSR) markers were used to evaluate levels of genetic similarity among *Coffea arabica* L. accessions from Tanzania and to estimate levels of genetic similarities in *C. arabica* and diploid coffee species. ISSR markers are considered to be more reproducible than RAPD markers due to high annealing temperature [103, 104] and have been used to measure genetic diversity in potato [101], barley [105], rice [106], finger millet (*Eleusine coracana*) [107], Sorghum [108] and Groundnut [109] as well as to identify cultivars in Maize [110], Wheat [111], potato [112], oilseed rape [113] and bean [114]. Huang et al. [115] also used ISSR markers to reveal genetic diversity and relationships in sweet potato and its wild relatives.

The ISSRs have several advantages for assessing genetic diversity [100] like no prior genomic information is required, abundantly polymorphic and reproducible; they are a good choice for detecting genetic diversity among crop species, germplasm characterization, establishment of genomic relation and molecular phylogeny. ISSRs analyses are more specific than RAPD analyses due to the longer SSR-based primers which enable higher-stringency amplification [60]. The high stringency reduces the problems with reproducibility, a common criticism against the low-stringency RAPD assay [116]. The shortcomings of ISSR markers like RAPDs are that most bands are scored as dominant markers giving no possibility to distinguish between homozygosis and heterozygosis directly. However, ISSR studies of natural populations have recently

demonstrated the hyper variable nature of these markers and their potential use in population-level studies [117, 118]. The technique allows for dissection below the subspecies level and this gives it a good level of applicability in the study of rare or endangered plants [59]. ISSRs have been used in conjunction with RAPD data to determine the colonization history of *Olea europaea* in Macronesia along with lineages in the species complex [119]; *Cicer* and cultivated chickpea [120]. The two techniques have also been utilized in examining the genetic diversity, varietal identification and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species [109].

1.5 Phytochemical and nutritional value of *C. spinosa*

In Ladakh, preparations from *Capparis* are used by the Amchis (Local traditional doctors) for treatment of common ailments. In Ayurveda given in splenic, renal and hepatic complaints; root bark of *Capparis spinosa* used as tonic, expectorant, anthelmintic, emmenegogue and analgesic. Many workers have worked on this plant for investigating both nutritional compositions and various bioactive compounds from different parts, presented in Table 1.3.

Table 1.3 Phytochemical characterization of *Capparis spinosa* L.

Germplasm	Bioactive compound isolated	Reference
Egypt	From the roots, indole glucosinolates like Glucoiberin, glucocapparin, sinigrin, glucocleomin, glucobrassicin, Neoglucobrassicin and 4-methoxyglucobrassicin were isolated.	Ahmed et al. [121]
Morocco	Occurrence of elemental sulphur and Methyl isothiocyanate, isopropyl isothiocyanate and sec-butyl isothiocyanates were extracted from flower buds.	Brevard et al. [122]
Iran	Methyl isothiocyanate, isopropyl isothiocyanate and sec-butyl isothiocyanate were extracted from unripe fruit and other parts.	Afsharypuor and Arefian. [123]

Isfahan (Iran)	Glucosinolates, Fatty acids, Sterol and Tocopherol were isolated from seed oil. It composed of linoleic, oleic and vaccenic acid as well as γ-tocopherol, δ-tocopherol and α-tocopherol. Sterol is also an important constituent of oil. Identified some sterols like sitosterol, campsterol, stigmasterol and Δ^{5}-avenasterol.	Matthaus and Ozcan , [124]
Argentina	The leaves have kaempferol, quercetin, isorhamnetin and their O-methyl derivative, thomnocitrin, rhamnetin and rhamnozin.	Juan et al. [125]
India	*p*-Methoxy benzoic acid was isolated from aqueous extract of aerial parts.	Gadgoli and Mishra, [126]
Turkey	Two glucose-containing 1*H*-indole-3-acetonitrile compounds, capparilosides A and B were isolated from mature fruits.	Calis et al., [127]
Turkey, Spain, Morocco, Italy and Greece	Quercetin 3-rutinoside, kaempferol 3-rutinoside and kaempferol 3-rhamnosyl-rutinoside as well as aglycones were evaluated from commercial capers.	Inocencio et al. [128]
Turkey	(6S)-hydroxy-3-oxo- α -aionol glucosides, together with corchoionoside C ((6*S*,9*S*) roseoside) and a prenyl glucoside were isolated from mature fruits.	Calis et al. [129]
Turkey	Glucocapperin, 4-hydroxyglucobrassicin, indole glucosinolate were extracted from the aerial parts.	Matthäus and Özcan, [130]
Island of Salina	Proximates and palmitic, stearic, oleic, linoleic and γ-linolenic acids as well as Flavanoid glycosides like, rutin, quercetin, kaempferol-3-rutinoside were isolated.	Giuffrida et al. [131]

Italy	Total phenolic compounds were extracted from buds.	Germano et al. [22]
Italy	Determined some flavonols (kaempferol and quercetin derivatives) and hydroxycinnamic acids (caffic, ferulic, p-cumaric and cinnamic acid).	Bonina *et al.*, [23]
Amman-Jordan	Two new compounds like β-sitosteryl glucoside-6'-octadecanoate and 3-methyl-2-butenyl-β-glucoside were isolated.	Khanfar et al., [132]
China	Eight Phenolics were isolated from the fruits by chromatographic methods and structure were established by spectroscopic methods as β-sitosterol, vanillic acid, p-hydroxybenzoic acid, protocatechuric acid, daucosterol, uracil, butanedioic acid and uridine.	Yu et al. [133]
China	Three new alkaloids capparispine, capparispine-26-O-β-d-glucoside and cadabicine 26-O-β-d-glucoside hydrochloride were isolated from the roots.	Romeo et al. [134]
China	Hypoxanthine and uracil were isolated from fruits.	Fu et al. [135]
China	Flavonoids and glycosides were isolated from roots and identified as kaempferol-7-rhamnosides, kaempferol-3-rutinoside, kaempferol-3-glucoside-7-rhamnoside, quercetin-3-rutinoside and isorhamnetin-3-7-dirhamnoside.	Fu et al. [136]
China	A dimeric 62-kDa lectin exhibiting a novel N-terminal amino acid sequence was purified from seeds.	Lam et al. [137]

China	A new antioxidant cappariside (4-hydroxy-5-methylfuran-3-carboxylic acid) together with four known organic acids were isolated.	Yang et al. [138]
China	Flavonoids, indols and phenolic acids were isolated from fruits.	Zhou et al. [139]
China	Seven known compounds were isolated and identified as: P-hydroxy benzoic acid, 5-(hydroxymethyl) furfural, bis(5-formylfurfuryl) ether, daucosterol, α-D-fructofuranosides methyl, uracil and stachydrine.	Feng et al. [140]
China	Two bioflavonoids, isoginkgetin and ginkgetin, together with three other flavonoids were isolated from fruits.	Zhou et al. [141]
Sicily	Rutin, isothiocyanates, total phenols, γ-tocopherol and Vit-C were measured from salted capers.	Tesoriere et al. [142]
Khuzestan	A qualitative and quantitative analysis of rutin from leaves, fruits and flowers was achieved and rutins were isolated from leaves, fruits and flower.	Ramezani et al., [143]
Dalmatia	Methyl isothiocynate was detected as major component of essential oil from leaves and flower buds.	Kulisic-bilusic et al. [144]
Tunisia	Carotenoids, Lutein, Violaxanthin, α-tocopherol, γ-tocopherol were isolated from leaves, buds and flowers.	Tlili et al. [145]
Tunisia	Sterols like phytosterol, β-sterol, campesterol, brassicasterol, Δ^{5}-avenasterol and stigmasterol were isolated.	Tlili et al. [146]

Tunisia	Phenolic compound like α-tocopherol, γ-tocopherol and Vit-C were extracted.	Tlili et al. [147]
Tunisia	Total storage protein, lipids, aliphatic alcohol, triterpinic alcohol, hexadecanol, octadecanol and tetracosanol were investigated. Beside these, citrostadienol, β-amyrin, gramisterol, cycloartanol and 2,4 methylcycloartenol were identified from seeds.	Tlili et al. [148]

1.6 Pharmacological activities

The plant C. spinosa has been utilized in preparations of various herbal formulations since long time for the treatment of various diseases. Most commonly it is used in liver problems, rheumatism, diabetes, etc. The details regarding the pharmacological importance of *C. spinosa* is mentioned in Table 1.4.

Table 1.4 Pharmacological/Bioactivities of *Capparis spinosa* L.

Germplasm collected from	**Bioactivity**	**Reference**
Bahrain	The alcoholic extract reported for anti microbial activity.	Mahasneh et al. [149]
Palestinian	The aqueous extracts showed antimycotic activity.	Ali-Shtayeh et al. [150]
India	It has been reported to possess hepatoprotective and antihepatotoxic activity against carbontetrachloride and paracetamol induced hepatotoxicity.	Gadgoli and Mishra [151]
Italy	The methanolic extract of flower buds showed *In vitro* antioxidant activity.	Germano et al. [22]
Italy	The methanolic extract of flowering buds possesses a strong antioxidant effectiveness as well as excellent photoprotection against	Bonina et al. Mishra [23]

	UVB-induced skin damage.	
Italy	Methanolic extracts of flowering buds showed antiallergic and antihistaminic activity.	Trombetta et al. [152]
Italy	Methanolic extract of buds possesses Antiviral and Immunomodulatory effect.	Arena et al. [153]
Morocco	Fruits were recognized as potent hypoglycemic agents.	Eddouks et al. [154]
Morocco	Aqueous extract of fruit exhibited hypolipidemic activity in normal and diabetic rats.	Eddouks et al. [155]
Morocco	Aqueous extract of evoked potent anti-hyperglycaemic and anti-obesity effect.	Lemhadri et al. [156]
Morocco	Aqueous extract showed Cardiovascular and hypotensive activity in spontaneously hypertensive (SH) rats.	Ali et al. [157]
Morocco	Aqueous extract possess Cardiovascular and *In vitro* vasorelaxant effect.	Zeggwagh et al. [158]
Iran	Aqueous extract of root bark exhibited hepatoprotective activity against CCl_4 induced hepatic damage in mice.	Aghel et al. [159]
Iran	Topical application of extract of flowering buds reduces UV-induced skin erythema in healthy human volunteers.	Khazaeli and Mehrabani [160]
Sicily	Hydrophilic extracts showed antioxidant activity.	Tesoriere et al. [142]
Turkey	Evaluated the genotoxic and antimutagenic effect of flower buds aqueous extracts on the *Allium cepa* L. root tips meristem cells.	Sultan and Celik [161]

China	A protein purified from seeds showed antiproliferative, antifungal and HIV-1 reverse transcriptase inhibitory activities.	Lam and Ng [162]
China	It protects against oxidative stress in systemic sclerosis dermal fibroblasts.	Cao et al. [163]
China	It exhibited anti-arthritic effect *in vivo*.	Feng et al. [140]
Dalmatia	It showed potent antioxidant activity.	Kulisic-bilusic et al. [144]
Tunisia	Leaves extract stimulate melanogenesis in a dose-dependent manner without cyto-toxicity by increasing tyrosinase protein expression and has the potential to be used as a possible tanning agent.	Matsuyama et al. [164]
Saudi Arabia	EtOAc fraction showed pronounced activity against *P. falciparum*$_{\text{GHA.}}$	Abdel-Sattar et al. [165]
Algeria	Antioxidant and antibacterial activities of methanolic extract of buds were investigated.	Bouriche et al. [166]

The present work is thus originated in view of the importance of *C. spinosa* to help the Indian pharmaceuticals, food and cosmetic industries to manufacture many products and drugs, to enhance the rate of seed germination, to understand genetic diversity among populations, to evaluate the antioxidant activity, to assess the nutritional content of flower buds of the populations of *C. spinosa* from the Ladakh region for commercial cultivation and realizing the existing lacunae in the study made so far in this aspect.

1.7 Objectives of the research works

- To optimize the conditions for breaking physical and physiological dormancy effectively to enhance the rate of seed germination of *C. spinosa* in Ladakh conditions for commercial propagation.

- To ascertain genetic diversity and population structure of *C. spinosa* based on DNA profiling techniques such as RAPD and ISSR in the trans-Himalayas, India for conservation strategies and future breeding program.

- To determine phytochemical compounds (phenol and flavonoid contents) and antioxidant activity of leaves collected from Ladakh region and their relationship with genetic diversity index.

- To analyze phytochemical composition (phenols and flavonoids) as well as antioxidant activity of the plant parts for pharmacological importance.

- To ascertain the nutritional composition (*viz.* minerals, vitamins and proximate content) of *C. spinosa* flower buds for formulation of nutritional supplements.

1.8 Outline of the works

Each of these pieces of work has distinct characteristics. At the same time they are related to one another. To clearly and coherently demonstrate the goal, results and conclusion of each piece of work, we have arranged each work chapter wise in a publishing format. The format will benefit readers to understand the idea of development, conclusion, coherence and full significance as each chapter will be a full manuscript from background to conclusion at publication stage.

CHAPTER 2

EFFECTS OF VARIOUS DORMANCY BREAKING TREATMENTS ON THE GERMINATION OF WILD CAPER (*CAPPARIS SPINOSA* L.) SEEDS FROM THE COLD ARID DESERT OF TRANS–HIMALAYAS

ABSTRACT

Very poor seed germination is the major problem in the wide scale cultivation of capers in the trans-Himalayan region. The present study was conducted to examine the role of various dormancy breaking treatments *viz*. soaking in the warm water, scarification, stratification, Conc. acids (H_2SO_4, HNO_3, and HCl), hormone (GA_3), Potassium Nitrate, alcohol, acetone, gamma ray irradiation and seed soaking in the cow urine on the germination of various caper seeds which was collected from wild plants growing in Ladakh. The seeds were left out in the germination chambers from 20 to 25 days after its treatments. Highest germination rate of 62% was observed when seeds were treated with H_2SO_4 for 40 min followed by 400 ppm of GA_3 soaking for 2 hrs. There are possibilities that hard seed coat was not the only hurdle to the germination and the dormancy was partly due to the physiological restrictions as well. It is recommended to germinate the caper seeds in the petridishes under specified treatments and then transfer these seedlings first in the greenhouse for 2-3 months and then to the open field conditions during spring-summer season (May-August) of Ladakh. The standardization of germination technique will ultimately help in the widespread cultivation of this wonder plant at the farmer's field of Ladakh.

2.1 INTRODUCTION

Capparis spinosa L. (Capparidaceae) also called 'Caper' and locally known as '*Kabra'* is one of the well known medicinal plant in '*Amchi system*' (local medicinal system) which is occasionally used by local people of Ladakh as a leafy vegetable and forage. It is an underutilized wild plant which grows in roadside, on the slopes, dry, rocky and stony soils, can withstand extreme temperature (–40 °C to +40 °C) of Ladakh and is highly drought tolerant [36]. In India, it is found in inner valleys of trans–Himalaya between 3020–3790 m which includes Indus, Nubra and Suru valleys of Ladakh region. This plant has multiple uses in cuisine as salad, leafy vegetable, pickle and condiments. Besides it brings many environmental benefits, including soil and water conservation, desertification control and land reclamation in fragile cold ecosystem of Ladakh [36]. Chemical studies have reported the presence of alkaloids, lipids, flavenoids and glucosinolates, caner preventing agents and biopesticides. Capers contain considerable amount of antioxidant bio–flavonoids '*rutin'* [22].

Caper plants can be propagated through seeds or stem cuttings; however, both methods present serious problems and limitations for the commercial propagation of this crop. Although capers are widely grown on dry and poor soil where environmental conditions are not easy for the cultivation of other crops, it is difficult to propagate seedlings because caper seed have both physical (seed coat) and physiological dormancy that led to difficulty in seed germination. Fresh caper seeds germinate readily, but in low percentages (1–2 %), whereas drying of seeds induces severe dormancy, which is difficult to overcome naturally [37]. As the dormancy in this crop is due to the hard seed coat, external treatments are necessary to overcome the prevailing dormancy. The structure of the seed and the mucilage which develops when the seed is placed in contact with water could impose an effective barrier against the diffusion of oxygen to the embryo [37].

Fruit weight, position on the mother plant and maturation stage also affects caper seed germination [167] besides this, an efficient method is standardized for ensuring

satisfactory seed germination by breaking the physical and/or physiological dormancy [168]. It has been reported that freshly harvested caper seeds presented the highest germination rate and the shortest time to reach 50% of the final percentage [169]. The effect of seed soaking treatments and soaking time individually or in combination was studied with the addition of GA to maximize the seed germination % [170]. The effects of temperature, light, pre soaking treatment and removal of seed coat have been reported to effect germination of various crops [171, 172]. For an example pre–chilling, scarification, and treatments with GA_3 or KNO_3 are the standard procedures used to enhance seed germination of dormant seeds [173]. To obtain higher germination (%) in Capparis various treatments were reported *viz.* gibberellic acid+KNO_3 [174], pretreatment with H_2SO_4 [175], H_2SO_4+GA_3 [176] and warm water + chilling [177].

Research studies on the seed germination of caper are scanty and insufficient across the world. Moreover, till date there is no report available about germination of caper seed from cold arid desert of Ladakh. Considering the potential uses of caper bush for rural development, as soil erosion control and water conservation agent, new crop and new income sources, present investigation was conducted to examine the importance of various dormancy breaking treatments that might affect germination of wild caper growing in Ladakh. The present study reports the outcome of preliminary investigations aimed at maximizing caper seed germination under cold arid desert of Ladakh.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and study site

The seeds of *Capparis* used in the present investigation were collected from the wild plants in Ladakh region where it grows abundantly. Ladakh is part of Indian Himalaya which is located at the latitude of 31° 44’ 57”– 32° 59’ 57” N and longitude of 76° 46’ 29” – 77° 41’ 34” E and at an altitude of 7,777–20,000 ft. (MSL), is characterized by diverse and complex land formations. The temperature ranges between +40 °C in summer to –40 °C during winter and annual precipitation is around 20–30 mm (mostly as snow) along with low relative humidity (20–30 %).

The dehisced fruits were collected in the month of October 2008 from different parts of Ladakh. The seeds were separated from the fruit material, rinsed in tap water thoroughly, dried in shade and kept at room temperature in polyethylene bags.

2.2.2 The average seed moisture content and 1000 seed weight

The average seed moisture content and 1000 seed weight is determined by moisture meter and digital weighing balance respectively.

2.2.3 Seed Viability Test

The seed viability was determined by Tetrazolium test using 500 seeds in five replications of 100 seed each [178]. The parts of the seed that are viable were become red or pink, the non–viable parts remained white.

2.2.4 Treatments and experimental design

The seed germination experiment was conducted as a completely randomized design with four replications. Each experimental unit was a petridish with 100 seeds. Prior to various treatment seed were surface sterilized by washing with 0.01% mercuric chloride, rinsed with distilled water, wash with 70% alcohol and finally rinsed with 3 washes of MilliQ (MQ) water. The surface sterilized seeds were used for the following treatments:

2.2.4.1 Acid treatments

Seeds were dipped in concentrated acids i.e. H_2SO_4, HCl and HNO_3 for 10–60 min at 10 min interval.

2.2.4.2 Gamma rays+H_2SO_4 treatments

Gamma rays irradiation of seeds at different doses (i.e. 10–50 KR at 10 KR interval) using the ^{60}Co gamma cell irradiator facility at the Botany Dept., RTM, Nagpur

University, Nagpur followed by dipping in conc. H_2SO_4 for 10–60 min at 10 min interval.

2.2.4.3 GA_3+H_2SO_4 treatments

Seeds were first pretreated in conc. H_2SO_4 for 20–60 min at 10 min interval, further dipped in GA_3 solutions (i.e. 100–500 ppm at 100 ppm interval) for a period of 1, 2, and 3 h.

2.2.4.4 KNO_3+H_2SO_4 treatments

Seeds were soaked at 3 different doses of KNO_3 (i.e. 0.1, 0.2 and 0.3 %) for a period of 6, 7 and 12 h after presoaking in conc. H_2SO_4 for 20, 30 and 40 min.

2.2.4.5 Scarification

Scarification of seeds by P320A sandpaper (sand grain/cm^2) then dipped in GA_3 solutions (i.e. 200–600 ppm at 100 ppm interval) for a period of 1, 2, and 3 h.

2.2.4.6 Stratification

Seeds were stratified at –20 °C for 1–30 days at 5 day interval.

2.2.4.7 Water treatments

Seeds were dipped in hot water at 50, 60 and 70 °C for 30–120 min at 30 min interval.

2.2.4.8 Alcohol and acetone treatments

Seeds were first soaked in absolute alcohol and acetone for 1, 2, 3, 4 and 5 days and then dipped in GA_3 solutions (100–600 ppm at 100 ppm interval) for a period of 1, 2, and 3 h.

All the treated seeds were placed in closed 9 cm petridishes (Ø 9 cm) which were lined with two sheets of filter papers Whatman No.1 and moistened with sterilized MQ water to which 250 ppm Carbendazim was added to prevent any fungal infection. Treated

seeds were placed on the moist paper for germination for 20–27 days and light was provided by Philips daylight lamps (324 µmol $m^{-2}s^{-1}$). A clear labeled lid was placed on top of each petridish denoting the treatment, temperature and replication. All these petridishes were then kept in seed germinator at 25±2 °C with relative humidity of 70 % and 20 h of light. Petri dishes were checked daily for germinated seeds and filter paper was moistened with sterilized MQ water as needed. Germination was determined by observing a visible radical or shoot. The number of seeds used for the germination tests were 4 replications x 100 seeds per replication for each treatment and was repeated twice.

2.2.5 Statistical analysis

Data given in percentages were subjected to arcsine (√X) transformation before statistical analysis. Mean seed germination percentage from various treatments were compared by one–way ANOVA using SPSS (Version 12.0) and differences between the means were compared by Duncan's multiple range test (DMRT). A probability of ≤0.05 was considered significant.

2.3 RESULTS AND DISCUSSION

The aim of the present investigation is to find the best treatment(s) so that this wonder plant can be commercially propagate at the farmer's field of Ladakh. It is the first report on germination studies of *Capparis spinosa* from cold arid desert of trans–Himalayan region of Ladakh. The average seed moisture content and 1000 seed weight as determined by moisture meter and digital weighing balance were found to be 7 % and 7.71 g respectively. The seed viability was found 79 % as determined by Tetrazolium test [178].

2.3.1 Concentrated acid (H_2SO_4, HCl and HNO_3) treatments

A comparison to observe the effect of various durations of conc. H_2SO_4 and HCl showed a positive effect of H_2SO_4 on seed germination while so significant germination was observed with conc. HCl. In case of conc. HNO_3 treatment there is no seed

germination at all. Maximum germination of 33.0±1.73 % was observed with 40 min of soaking in conc. H_2SO_4 (Table 1). This result is in agreement with that of Lyons *et al.* [179] where they observed seed germination of 33 % when seeds were soaked conc. H_2SO_4 for 40 min. However, any increase or decrease in acid soaking time significantly reduces the seed germination which can be attributed to embryo damage. Poor germination or no germination in case of conc. HCl and HNO_3 respectively might be due to the inability of these treatments to break the physical dormancy.

Table 2.1 Effect of soaking durations of concentrated acids (H_2SO_4 and HCl) on seed germination (%).

Soaking duration (min)	Concentrated Acids	
	H_2SO_4	HCl
10	$20.67^{ab} \pm 1.76$	4.33 ± 0.67
20	$23.33^{b} \pm 1.20$	4.67 ± 0.33
30	$27.67^{c} \pm 0.77$	6.00 ± 1.00
40	$33.00^{d} \pm 1.73$	6.33 ± 1.20
50	$20.67^{ab} \pm 0.77$	7.67 ± 0.77
60	$19.00^{a} \pm 0.57$	5.33 ± 0.33
Mean germination (%) ± SEM	24.22 ± 1.29	5.72 ± 0.39

$^{a, b, ab, c, d}$ means bearing different superscripts in a column differ significantly ($P < 0.01$)

2.3.2 KNO_3+H_2SO_4 treatments

Pre–treatment with H_2SO_4 for 15–30 min was found to be an effective method to increase germination [175]. While, seed treatments with KNO_3 or GA_3 is known to improve the germination percentage. When H_2SO_4 pretreated seeds were treated with KNO_3 in different combinations, the highest germination of 61 % (Table 2) was achieved in case of presoaking in conc. H_2SO_4 (for 20 min) followed by dipping in KNO_3 (0.2 % for 7 h). Any increase or decrease in the concentration of KNO_3 or soaking duration along with further increase in the presoaking time has negative effect on the overall germination (%).

Germination percentage of 44.3 % was found in seed soaked in 0.3 % KNO_3 for 7 h along with 20 min H_2SO_4 presoaking. Yahyaoglu [180] proposed that seeds should be

soaked in 0.2 % KNO_3 for better germination. In present study average seed germination percentage of 35.33±6.70 in 0.2 % KNO_3 was higher than the 0.1 and 0.3 % KNO_3. Beside this in the application of KNO_3, duration of 7 h increased the germination percentage of the seeds over other durations i.e. 6 and 12 h (Table 2).

Table 2.2 Effect of presoaking treatments of concentrated H_2SO_4 (duration) in combination with KNO_3 (concentrations and durations) on germination (%).

H_2SO_4 presoaking duration (min)	KNO_3 concentration (%)									Mean germination (%)±SEM
	0.1			0.2			0.3			
	Soaking duration (h)			Soaking duration (h)			Soaking duration (h)			
	6	7	12	6	7	12	6	7	12	
20	37.0	12.0	12.7	27.0	61.0	13.0	34.0	44.3	13.0	27.3^{b}±3.25
30	32.0	36.0	17.0	36.0	29.0	23.0	37.0	30.0	16.0	27.7^{b}±1.51
40	7.0	11.0	13.0	21.0	16.0	11.0	21.0	9.0	12.0	13.6^{a}±0.96
Mean germination(%)	26.00^{xyz}	19.67^{wxy}	14.56^{wx}	27.00^{yz}	35.33^{z}	15.67^{wx}	31.00^{yz}	27.77^{yz}	13.67^{w}	-
±SEM	±4.62	±4.13	±1.09	±2.29	±6.70	±1.93	±2.61	±5.20	±0.94	-

$^{a, b, c}$ different superscripts in a column differ significantly ($P < 0.01$)
$^{w, x, y, z}$ different superscripts in a row differ significantly ($P < 0.01$)

2.3.3 GA_3+H_2SO_4 treatments

Gibberellic acid (GA) is known to play an essential role in seed germination, stem elongation and flower development [174]. The highest seed germination (62 %) was obtained for presoaking in sulfuric acid (40 min) followed by dipping in 400 ppm GA_3 for 2 h (Table 3). Lesser or longer treatment time was inhibitory in each case. However for 300 and 500 ppm GA_3 treatment, the highest germination of 47 and 45% was obtained after 2 h soaking in GA_3 along with 40 min of H_2SO_4 presoaking treatment. This means that the regulation of endogenous GA levels after seed imbibitions along with specific H_2SO_4 presoaking time (i.e. 40 min) is crucial factor in determining the seed germination. High germination percentages were obtained using conc. H_2SO_4, followed by a 90 min soaking in 0.01 % GA_3 solution [176].

Table 2.3 Effect of concentrated H_2SO_4 presoaking (durations) along with GA_3 (concentrations and durations) on germination (%).

GA_3 (ppm)	Duration (h)	H_2SO_4 presoaking duration (min)					Mean germination (%) SEM±
		20	30	40	50	60	
100	1.0	7.0	31.0	33.0	29.0	5.0	21.2±3.25
	2.0	10.0	34.0	39.0	32.0	7.0	24.4±3.54
	3.0	7.0	27.0	31.0	24.0	6.0	19.0±2.75
200	1.0	11.0	33.0	37.0	32.0	11.0	25.0±3.13
	2.0	12.0	37.0	41.0	37.0	13.0	27.0±3.46
	3.0	17.0	27.0	33.0	27.0	9.0	22.7±2.35
300	1.0	12.0	39.0	42.0	34.0	15.0	27.4±3.36
	2.0	14.0	43.0	47.0	39.0	16.0	31.7±3.77
	3.0	7.0	35.0	39.0	31.0	11.0	24.7±3.44
400	1.0	16.0	42.0	47.0	39.0	15.0	32.0±3.71
	2.0	7.0	47.0	62.0	42.0	17.0	35.0±5.42
	3.0	11.0	37.0	44.0	36.0	12.0	27.0±3.72
500	1.0	14.0	34.0	39.0	35.0	13.0	27.0±3.04
	2.0	15.0	39.0	45.0	37.0	14.0	30.2±3.53
	3.0	10.0	29.0	36.0	32.0	11.0	23.6±2.94
Mean germination (%) ± SEM		11.47 ±0.55	35.67 ±0.77	41.13 ±1.15	33.70 ±0.77	11.67 ±0.57	

Presoaking with H_2SO_4 ($P<0.01$); GA_3 ($P<0.01$); Duration ($P<0.01$); Presoaking with H_2SO_4 x GA_3 ($P<0.01$); Presoaking with H_2SO_4 x Duration ($P<0.01$); GA_3 x Duration ($P=0.642$); Presoaking with H_2SO_4 x GA_3 x Duration ($P<0.01$)

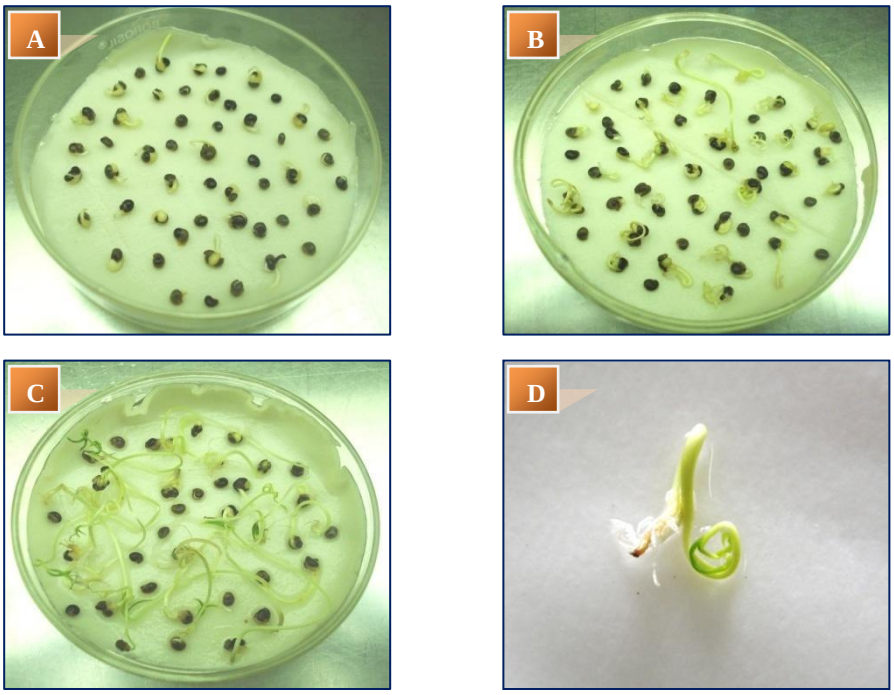


Figure 2.1 Effect of conc. H_2SO_4 (40 min.) and 400 ppm GA_3 (2 hr.) on caper seed germination percentage.

It is proposed that disruption of seed coat allowed diffusion of Oxygen that interact with growth regulators and removed seed dormancy. Our results are in agreement with that Soyler and Khawar [41], who found that the seed dormancy is mainly due to the seed coat that prevents germination. He observed that when the seeds get in touch with water, mucilage comes into existence on the coat and hinders embryo to take O_2 preventing germination. He further points out that GA_3 has positive effect on germination. However, the relationship between GA_3 and O_2 is not known. Probably, GA_3 decreases the O_2 need for the germination.

2.3.4 Gamma rays+H_2SO_4 treatments

Positive effect of gamma rays irradiation on seed germination is already known in many agricultural crops [181]. Therefore, *Capparis* seeds were also treated with different doses of gamma rays along with conc. H_2SO_4 treatment for different durations. Maximum germination of 26% was observed when seeds were treated with 30 KR gamma rays followed by 40 min conc. H_2SO_4 treatment. Any further increase or decrease in the gamma rays dose or acid duration is having negative effect on the overall germination percentage (Table 4).

Table 2.4 Effect of Gamma rays (doses) in combination with concentrated H_2SO_4 (durations) on germination (%).

Gamma rays (KR)	H_2SO_4 soaking duration (min)			Mean germination (%)±SEM
	20	40	60	
10	7.0	17.0	10.0	11.67^{a}±1.76
20	9.0	19.0	13.0	13.67^{b}±1.57
30	17.0	26.0	19.3	20.77^{d}±1.57
40	14.0	23.0	17.0	17.00^{c}±1.37
50	11.0	21.0	13.0	15.00^{b}±1.57
Mean germination (%)	11.60^{x}	21.40^{z}	14.47^{y}	
±SEM	1.06	0.91	0.95	

$^{a, b, c}$ different superscripts in a column differ significantly ($P < 0.01$)
$^{x, y, z}$ different superscripts in a row differ significantly ($P < 0.01$)

2.3.5 Other treatments

Scarification, stratification and hot water treatments were found ineffective to break seed coat dormancy. Application of acetone, alcohol and HNO_3 although broke the seed coat but there was no further germination.

Low germination percentage in case of control was possibly due to the seed coat of the capers that forms mucilage on soaking in water. The mucilage surrounding the seed is supposed to inhibit diffusion of Oxygen to the embryos and prevent germination. Sulfuric acid treatment to remove mucilage and soaking in either of KNO_3, GA_3 or gamma ray treatment was found effective to allow penetration of Oxygen from the surroundings to the embryos and increased germination of seeds. Germination in each case was superior over the control (0–2 %).

When a comparison is made between GA_3, KNO_3 and gamma ray treatments along with H_2SO_4 pretreatment, we found GA_3 is in general superior to KNO_3 and is superior over gamma ray treatment in breaking seed dormancy. But the best germination values were almost similar in both the treatments (i.e. GA_3 and KNO_3).

There are possibilities that hard seed coat was not the only hurdle to the germination and the dormancy was partly due to the physiological restrictions as well. As germination in caper is controlled by both physical and physiological factors, pre–sowing treatments are necessary to overcome the dormancy. Acid scarification followed by addition of GA_3 or KNO_3 solution to the germination substrate is a simple, efficient and cost effective method for ensuring satisfactory seed germination. The described procedure could help in improved germination of seeds in pots or nurseries before transplanting seedlings to the desired places in the field.

2.4 CONCLUSION

In conclusion, it is recommended to germinate the caper seeds in the petridishes under specified treatments and then transfer these seedlings first in the greenhouse for 2–3 months and then to the open field conditions during spring–summer season (May–August) of Ladakh. It is speculated that under extreme environmental circumstances of Ladakh, the seed coat may not be disrupted due to the action of soil bacteria. The described method will ultimately help in the widespread cultivation of this wonder plant at the farmers' field of Ladakh. Further experiments will continue to study the effect of time between fruit harvest and seed germination especially under cold arid desert of Ladakh.

CHAPTER 3

GENETIC VARIABILITY STUDIES AMONG THREE NATURAL POPULATIONS OF *CAPPARIS SPINOSA* L. FROM COLD ARID DESERT OF TRANS-HIMALAYAS USING DNA MARKERS

ABSTRACT

For the assessment of genetic relationships among the genotypes of *Capparis spinosa* grow naturally from the Ladakh region, we have collected 90 genotypes from nine sampling sites localized from three valleys at different altitudes *viz*. Suru (3309 m), Nubra (2926 m) and Indus (3505 m) of trans-Himalayan region. Molecular characterization of the genotypes was carried out using 40 PCR markers (20 RAPDs and 20 ISSRs). RAPD analysis yielded 223 fragments, of which 220 (98.65%) were polymorphic, whereas ISSR produced 85 bands, of which all are found polymorphic, demonstrating high genetic variations among the genotypes. ISSR markers were found more efficient with regards to polymorphism detection. The genotypes were clustered (using neighbor joining method) more likely as per their collection sites (populations) based on RAPD, ISSR and in combination of RAPD and ISSR data with high bootstrap values (> 70%). Statistically unbiased clustering of genotypes without prior knowledge of populations distributed 90 genotypes more likely as per their populations with high likelihood probability score. The genetic diversity was high among the genotypes (Nei's genetic diversity = 0.454 and Shannon's information index = 0.643) as measured by combination of both RAPD and ISSR markers. Molecular variance measured revealed existence of high variation among genotypes (60.47%), whereas the variance among populations was (33.61%) and among valleys was (5.90%) using the combine dataset of RAPD and ISSR. Pertaining to the management of caper, the high genetic differentiation of population indicates the requisite of conserving the utmost possible number of populations from different valleys of trans-Himalayas.

3.1 INTRODUCTION

Fragmented distributions of plant populations are caused not only by human activity but also by natural factors, such as long term, large-scale climate oscillations, topographical changes, the isolation of suitable habitats, or other ecological changes. Habitat fragmentation is a significant threat to the maintenance of biodiversity [182] which is expected to reduce genetic diversity and increases inter population genetic divergence [183]. However, habitat fragmentation does not always lead to reduced genetic variation [184] but it may increase the genetic diversity of a fragmented population than continuously distributed population [185]. This is because the effects of habitat fragmentation on genetic diversity and population structure can be affected by other factors, such as population size, gene flow and the time scale of fragmentation [186]. Studies of the genetic diversity of naturally fragmented populations may not only reveal the ecological consequences of population fragmentation over long periods of time but also provide a frame of reference for predicting the consequences of habitat fragmentation by human activities [184].

Capparis spinosa L. (Capparidaceae) also called 'Caper' and locally known as '*Kabra'* is one of the oldest known medicinal plant in 'Amchi system' (local medicinal system) which is occasionally used by local people of Ladakh as a leafy vegetable and forage. In India, it is found in inner valleys of trans–Himalaya between 3020–3890 m which includes Indus, Nubra and Suru valleys of Ladakh region. This plant has multiple uses in cuisine as salad, pickle and condiments. Bio-chemical studies have reported the presence of alkaloids, lipids, flavenoids and glucosinolates, cancer preventing agents and biopesticides in *Capparis spinosa* [36].

C. spinosa is certainly much more threatened since its habitat has been extremely destroyed, mainly by anthropogenic action, and its distribution has been reduced to a very restricted area. Earlier, caper leaves were used only in small quantities by local people for vegetable purpose but, recently commerce and demand have increased. Heavy extraction from the wild along with heavy grazing at high altitude pasture in the trans-Himalayan

region of Ladakh has now threatened its survival. Since ages, the caper which is growing wild in Ladakh has developed considerable variability. The genotypes which grow at Ladakh are area specific, suitably adapted for survival since it grows at high altitudes (3,000-4,000 m above mean sea level) along with temperature and nutrient stress that they are subjected under the cold arid environment.

PCR based marker system like random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) have been used extensively both for DNA fingerprinting [187] and population genetic studies [188]. Limited reports are available on the molecular characterization of caper [46, 47]. The reduced number of *Capparis* individuals in trans-Himalayas makes the species highly susceptible to extinction, and conservation measures should be implemented immediately. Information on levels and distribution of genetic diversity is essential for successful management and preservation of populations of threatened species.

In this study, we used RAPD and ISSR analysis to assess the genetic diversity, with the aim to determine population differentiation and structure of isolated populations of *Capparis spinosa* from three valleys across its distribution in the trans-Himalayan range of India in a scenario of local adaptation at high altitudes along with providing insight to facilitate conservation management of these populations.

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials

Ninety wild grown individual plants collected from nine different locations were obtained from three valleys (Indus, Nubra and Suru) with altitude ranging from 3135 m (Nubra), 3309 m (Suru) to 3435 m (Indus) from the cold arid desert of trans-Himalayas (Fig. 3.1) during 2009-2010. The detail of the number of genotypes collected from different sampling site is included in Table 3.1. The interval between samples was 100-500 m, the pair wise distance between populations was 5–35 Km, whereas, the pair wise distance between valley divisions was 50-250 Km.

Table 3.1 Ninety genotypes of *C. spinosa* collected from nine populations covering three valleys of Trans-Himalayan region of Ladakh.

Valleys	Site (Villages)	Latitude (ºE)	Longitude (ºN)	Altitude (Meter)	Samples
Suru	Batalik	76.2465	34.5541	3309	1-8
Indus	Nimmu	77.3434	34.1926	3319	9-12
	Basgo	77.2917	34.2139	3241	13-16
	Phyang	77.4669	34.1615	3347	1 7-31
	Phey	77.4815	34.1399	3185	32-46
	Thiksey	77.6648	34.0554	3435	4 7-62
Nubra	Skampuk	77.4158	34.6383	3197	63-68
	Skuru	77.3685	34.6485	3135	69-78
	Tirchey	77.3513	34.6579	3159	79-90

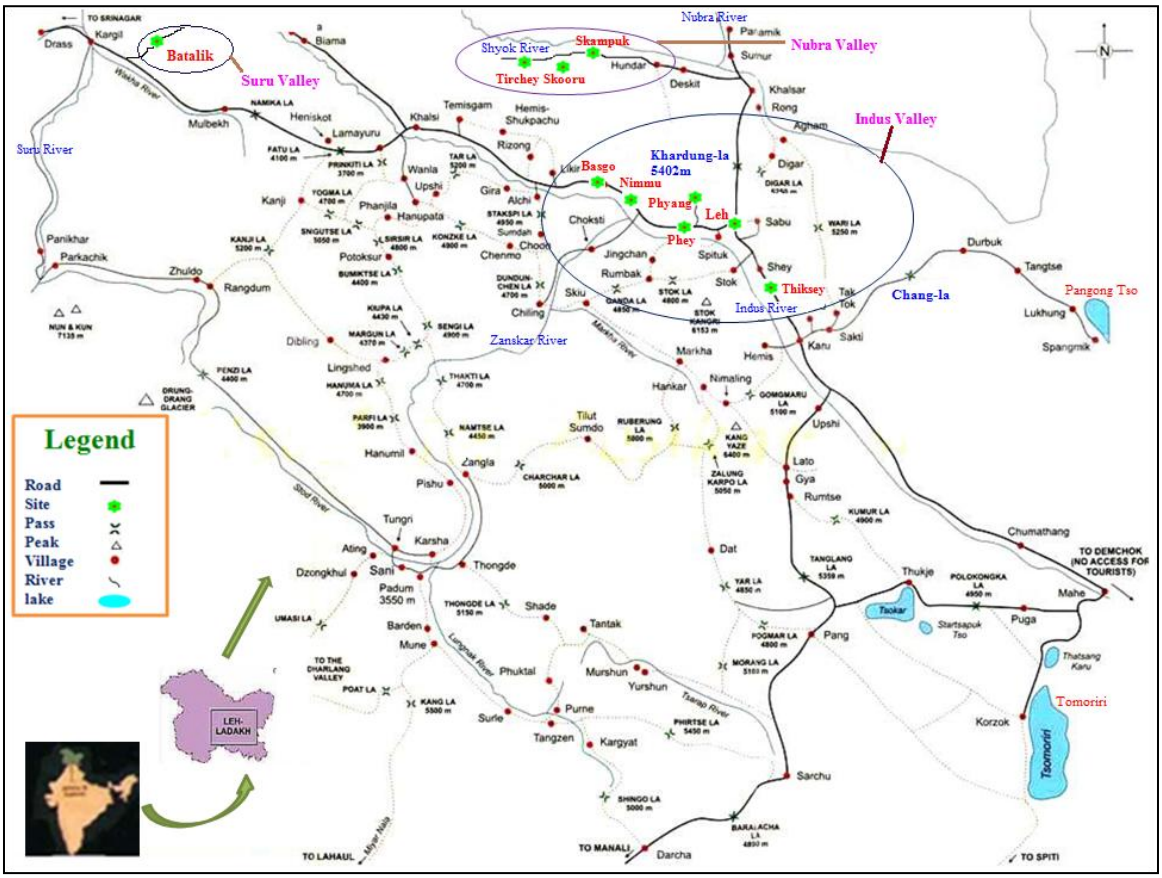


Figure 3.1 Collection sites of 90 *Capparis* individuals from three valleys (Indus, Suru and Nubra) and nine villages located in Ladakh (Jammu & Kashmir, India)

3.2.2 DNA extraction and PCR amplification

Total genomic DNA was extracted from frozen leaves (5 g) by the CTAB method [189] with minor modifications. All the experiments were repeated 3-4 times to check reproducibility.

3.2.3 RAPD analysis

Twenty random decamer primers from IDT Tech, USA (Table 3.2) were used for RAPD amplification following the protocol of Williams et al. [190]. Amplification reaction were performed in volumes of 25 µl containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTPs, 0.4 µM primer, 20 ng template DNA and 0.5 unit of *Taq* polymerase ('Sigma-Aldrich, USA'). The first cycle consisted of denaturation for 5 min. at 94°C, primer annealing at 37°C for 1 min. and extension at 72°C for 2 min. In the next 40 cycles denaturation period is 1 min. at 92°C, while annealing and extension parameters remained same as in the first cycle while final extension step at 72°C for 7 min.

Table 3.2 List of primers used for RAPD amplification, GC content, total number of loci, the level of polymorphism and resolving power.

Primer	Nucleotide sequence (5'-3')	Tm (°C)	Total number of loci	Percentage of polymorphic loci	Total number of fragments amplified	Resolving Power
S-21	CAGGCCCTTC	36.4	07	100.00	426	9.46
S-22	TGCCGAGCTG	40.7	16	100.00	802	17.82
S-23	AGTCAGCCAC	34.3	13	92.30	952	21.15
S-24	AATCAGCCAC	30.1	14	100.00	992	22.04
S-25	AGGGGTCTTG	32.6	12	100.00	599	13.31
S-26	GGTCCCTGAC	35.2	11	90.90	546	12.13
S-27	GAAACGGGTG	33.2	14	92.85	703	15.62
S-28	GTGACGTAGG	31.1	09	100.00	339	7.53
S-29	GGGTAACGCC	37.4	10	100.00	486	10.8
S-30	GTGATCGCAG	33.1	11	100.00	580	12.8
S-31	CAATCGCCGT	36.7	06	100.00	323	7.17
S-32	TCGGCGATAG	34.0	05	100.00	219	4.86
S-33	CAGCAGCCAC	37.7	13	100.00	818	18.17
S-34	TCTGTGCTGG	34.3	11	100.00	634	14.08
S-35	TTCCGAACCC	34.2	10	100.00	502	11.15
S-36	AGCCAGCGAA	38.3	12	100.00	646	14.35
S-37	GACCGCTTGT	35.7	14	100.00	357	7.93
S-38	AGGTGACCGT	36.2	12	100.00	659	14.64
S-39	CAAACGTCGT	34.2	11	100.00	484	10.75
S-40	GTTGCGATCC	33.5	12	100.00	675	15.00
	Total	-	**223**	**98.65**	**11742**	-

3.2.4 ISSR analysis

Twenty ISSR primers were obtained from 'Applied Biosciences, India' (Table 3.3) and PCR amplification was performed in reaction cocktail similar to RAPD. Initial denaturation for 5 min. at 94°C was followed by 40 cycles of 1 min. at 94°C, 1 min. at specific annealing temperature (± 5°C of Tm), 2 min. at 72°C and a 10 min. final extension step at 72°C for 7 min. PCR products were stored at 4 °C before analysis.

Table 3.3 List of ISSR primers used, sequence information, GC content, total number of loci, the level of polymorphism, size range of fragments and resolving power. Where, (Y = C, T; R = A, G).

Primers	Nucleotide sequence	G+C (%)	Total number of loci	Number of Polymorphic loci	Percentage of polymorphic Loci	Total no. of bands amplified	Resolving power
ISSR 1	$(AG)_8T$	47.0	16	16	100	797	17.71
ISSR 2	$(AC)_8T$	47.0	08	08	100	205	4.55
ISSR 3	$(TG)_8A$	47.0	05	05	100	56	1.24
ISSR 4	$(AG)_8YT$	47.2	10	10	100	407	9.04
ISSR 5	$(GA)_8YT$	47.2	13	13	100	509	11.31
ISSR 6	$(GT)_8YC$	52.7	16	16	100	735	16.33
ISSR 7	$(ACC)_6$	66.6	12	12	100	807	17.93
ISSR 8	$(GGC)_6$	100	05	05	100	279	6.20
Total	-	-	**85**	**85**	**100**	**3795**	-

ISSR 9-20 did not amplify with the genotypes used in the present investigation. Individual primers sequences were given in the parentheses. ISSR 9 $[(AT)_8T]$; ISSR 10 $[(TA)_8RT]$; ISSR 11$[(AT)_8YA]$; ISSR 12 $[(CT)_8 T]$; ISSR 13 $[(TC)_8 A]$; ISSR 14 $[(GT)_8 A]$; ISSR 15 $[(TGC)_6]$; ISSR 16 $[(TGCA)_4]$; ISSR 17 $[(CTAG)_8]$; ISSR 18 $[(GA)_8 T]$; ISSR 19 $[(CT)_8 RA]$ and ISSR 20 $[(CCG)_8]$

3.2.5 Agarose gel electrophoresis

Amplification products were electrophoresed on 1.5% agarose gel (Life Science Technologies, USA) and run at constant voltage (50V) in 1X TBE for approximately 2 hr, visualized by staining with ethidium bromide (0.5µg ml^{-1}). After electrophoresis, the gels were documented on a gel documentation system (Alpha Innotech, Alphaimager, USA).

3.2.6 Data collection and analysis

The banding patterns obtained from RAPD and ISSR were scored as present (1) or absent (0), each of which was treated as an independent character. Jaccard's dissimilarity coefficient (J) was calculated, subjected to cluster analysis by bootstrapping and neighbor-joining method using the program DARWIN (version 5.0.158). POPGENE was used to calculate within species diversity (Hs), total genetic diversity (Ht) and Nei's unbiased genetic distance among different individuals. Data for Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the nine populations were also analyzed [191].

The RAPD and ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA) [192], using three hierarchical levels; individual, population and their regions. GenAlEx was used to calculate a principal coordinates analysis (PCA) that plots the relationship between distance matrix elements based on their first two principal coordinates [193]. According to Prevost and Wilkinson [112] the resolving power (Rp) of a primer is: Rp=Σ*IB* where *IB* (band informativeness) takes the value of: 1–[2* (0.5–P)], P being the proportion of the 90 individuals containing the band.

In order to determine the utility of each of the marker systems, diversity index (DI), effective multiplex ratio (EMR) and marker index (MI) were calculated according to Powell *et al.* [194]. DI for genetic markers was calculated from the sum of the squares of allele frequencies: $DI_n = 1- \Sigma pi^2$ (where `pi' is the allele frequency of the i^{th} allele). EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay. EMR (E) = n_p (n_p/n). MI is defined as the product of the average diversity index for polymorphic bands in any assay and the EMR for that assay, MI = DI_{avp} * E. Wright's inbreeding coefficient (Fst) was also calculated using the programme *AFLP-SURV* [195].

Additionally we performed Bayesian clustering analyses using STRUCTURE v2.2 [196, 197] to infer the number of cluster (K) and the likelihood probability of

individual genotype assigned to each cluster without prior knowledge of populations. Parameter sets assumed the admixture model with alleles correlated among patches. We executed analyses with no a-priori information on patch structure. Nine independent simulations were run, with a 100,000 burn-in period length, testing from one to nine clusters (K 1–9) for each. We calculated the average of each K likelihood values through all runs as well as ΔK statistics [198] to verify the correct number of segregate groupings.

3.3 RESULTS AND DISCUSSION

3.3.1 Genetic variability details from RAPD markers

All the chosen primers yielded 11742 fragments which range from five (S32) to sixteen (S22). Out of 223 amplified bands, 200 (98.65%) were found polymorphic, with average numbers of polymorphic bands per primer as 11.0 (Table 3.2). The amplified DNA fragments are within 100 bp to 1000bp. The representative gel photograph using RAPD primers is shown in Figure 3.2. Genetic diversity measured in terms of H, I, Ht, NPL and PPL for valley divisions were found higher for Nubra valley and least for Suru valley indicating more variability in Nubra valley and least in Suru valley (Table 3.4).

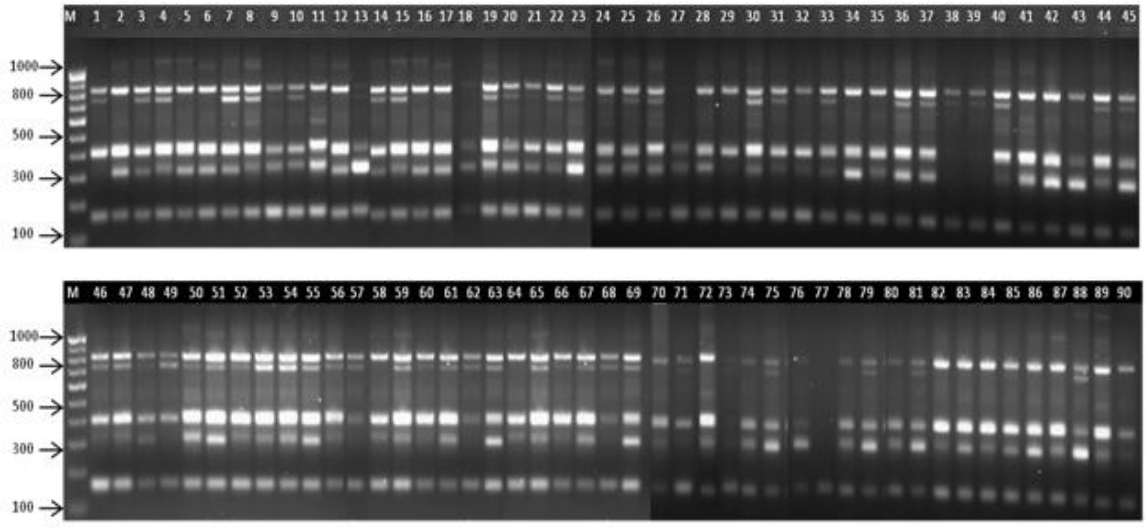


Figure 3.2 DNA profiling of *C. spinosa* on agarose gel, amplified with RAPD primer **S21**. 1. Batalik (1-8); 2. Nimmu (9-12); 3. Basgo (13-16); 4. Phyang (17-31); 5. Phey (32-46); 6. Thiksey (47-62); 7. Skampuk (63-68); 8. Skuru (69-78); 9. Tirchey (79-90). M = the size of molecular markers in base pairs using λ DNA.

The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the *Capparis* genotypes. The resolving power of the 20 RAPD primers ranged from 7.17 for primer S31 to a maximum of 22.04 for primer S24.

3.3.2 RAPD derived dendrogram analysis

A dendrogram analysis based on bootstrapping and neighbor joining (NJ) method grouped all the 90 genotypes into five main clusters (Figure 3.3), whereas at populations level all the 90 genotypes were clustered with bootstrap support value of > 70% (Figure 3.4a). Cluster I contains individuals from Batalik (Suru), Nimmu and Basgo (Indus) villages, cluster II represents individuals from Phey and Phyang (Indus). However, cluster III and IV contains individuals from Skuru and Tirchey (Nubra), clusters V have the individuals from Thiksey (Indus) and Skampuk (Nubra). An unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the populations suggested that the genotypes were more likely distributed (at high likelihood probability) with respect to their geographical distribution albeit small interference (Figure 3.4b).

Table 3.4 Summary of genetic variation statistics for all loci of RAPD, ISSR and RAPD+ISSR among the *Capparis* populations with respect to their distributions among three valleys

Marker Type	Sample size	H	I	Ht	PPL
RAPD					
Suru	8	0.239±0.196	0.358±0.277	0.239±0.038	65.92
Indus	54	0.35±0.142	0.52±0.18	0.350±0.02	96.86
Nubra	28	0.354±0.157	0.52±0.205	0.354±0.025	93.27
Mean	-	0.315	0.466	0.315	-
ISSR					
Suru	8	0.421±0.11	0.603±0.143	0.421±0.012	96.3
Indus	54	0.487±0.017	0.680±0.017	0.487±0.001	100
Nubra	28	0.460±0.044	0.651±0.047	0.460±0.002	100
Mean	-	0.456	0.645	0.456	-
RAPD+ISSR					
Suru	8	0.401±0.119	0.581±0.15	0.401±0.014	96.71
Indus	54	0.478±0.024	0.671±0.024	0.478±0.001	100
Nubra	28	0.483±0.022	0.676±0.023	0.483±0.001	100
Mean	-	0.454	0.643	0.454	-

H = Nei's gene diversity; I = Shannon's Information index; Ht = Heterogeneity; PPL = Percentage polymorphic loci; Hs = Genetic diversity in population was found nil while, Gst = Genetic diversity between population was observed 1.0 in all the cases

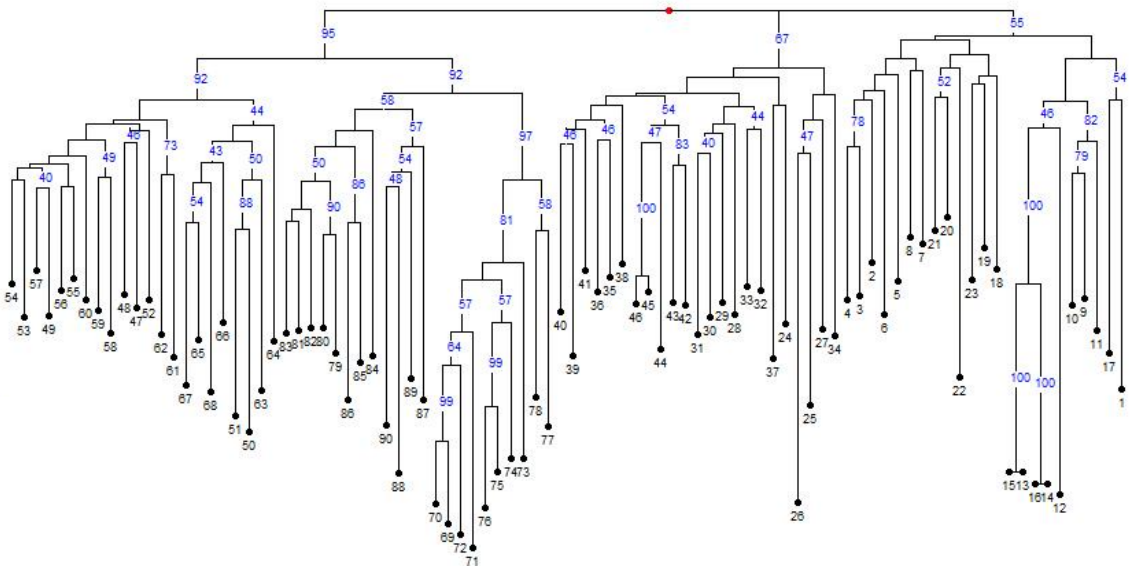


Figure 3.3. Dendrogram generated by Neighbor joining (NJ) clustering technique showing relationships between 90 genotypes of *C. spinosa* collected from 9 different populations, *viz.* Batalik (1-8); Nimmu (9-12); Basgo (13-16); Phyang (17-31); Phey (32-46); Thiksey (47-62); Skampuk (63-68); Skuru (69-78); Tirchey (79-90) based on RAPD profiling. Number indicates bootstrap support values.

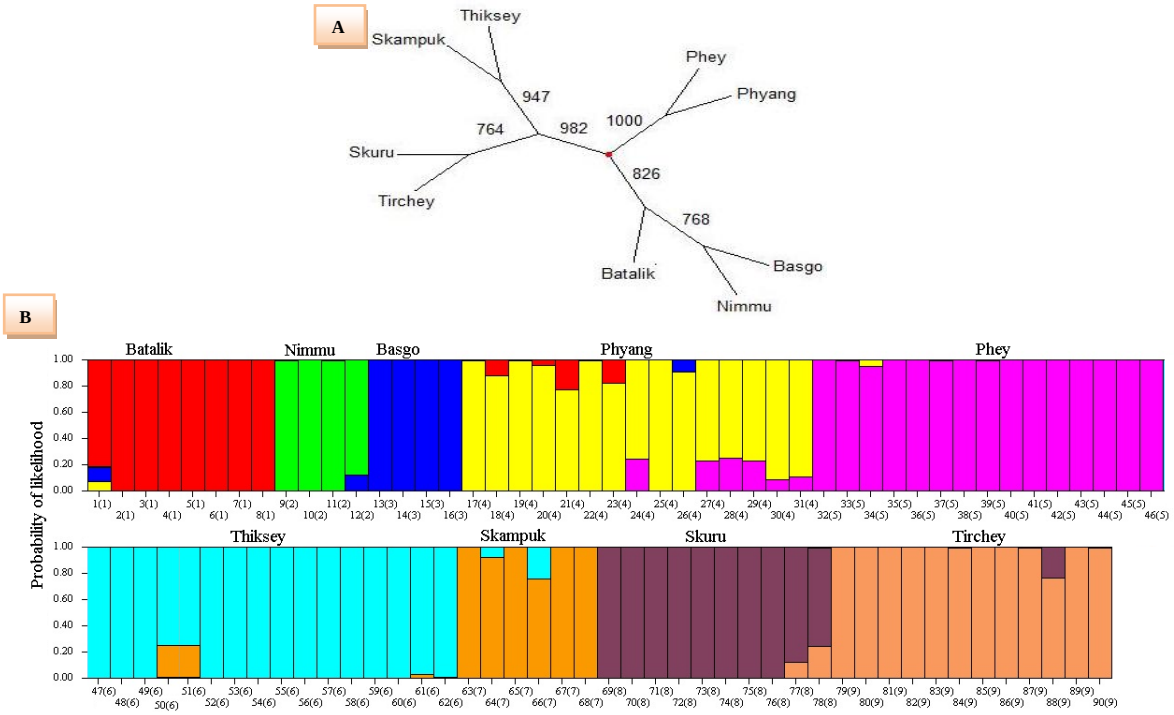


Figure 3.4. (A) NJ tree representing clustering of genotypes at populations' level along with supported bootstrap values, (B) Statistically unbiased clustering of 90 genotypes at population level. Genotypes from different populations are represented with different colours: Batalik (red), Nimmu (green), Basgo (blue), Phyang (yellow), Phey (pink), Thiksey (light blue), Skampuk (brown), Skuru (deep brown) and Tirchey (light brown).

3.3.3 Genetic variability details from ISSR markers

Out of 20 ISSR primers used, only eight amplified which produced average 85 bands, of which all were found polymorphic. Number of amplified fragments varied from five (ISSR3 and 8) to sixteen (ISSR1 and 6) and both average numbers of bands and polymorphic bands per primer is 10.62 (Table 3.3; Figure 3.5).

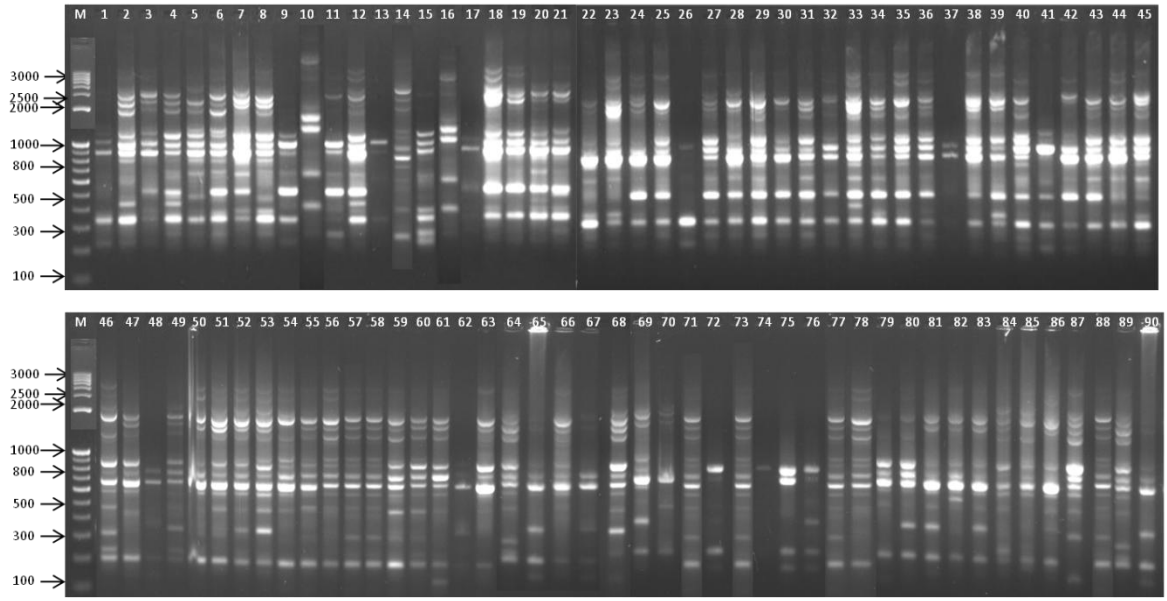


Figure 3.5 Profile of *C. spinosa* on agarose gel, amplified with ISSR primer S2. 1. Batalik (1-8); 2. Nimmu (9-12); 3. Basgo (13-16); 4. Phyang (17-31); 5. Phey (32-46); 6. Thiksey (47-62); 7. Skampuk (63-68); 8. Skuru (69-78); 9. Tirchey (79-90). M = the size of molecular markers in base pairs using λ DNA.

The sequences of these 20 primers seem to indicate that microsatellites more frequent in caper contain the repeated di-nucleotides (AG)n, (AC)n, (TG)n, (GA)n, (GT)n, and tri-nucleotides (ACC)n, and (GGC)n. The number of bands produced with different repeat nucleotide were more with the (AG)nT, (GT)nYC and (ACC)n primers (ISSR1, 6 and 7). In the present investigation, the primers that were based on the (AG)n, and (GT)n motif produced more polymorphism (16 bands per primer) than the primers based on any other motifs. However, (AT)n and some other primers gave no amplification products (Table 3), despite the fact that (AT)n di-nucleotide repeats are thought to be the most abundant motifs in plant species [187, 199]. Possible explanation could be that ISSR primers having (AT) or (TA) motifs due to sequence complementarity

may form dimers during PCR amplification [59] or it may not be annealing with template DNA due to low Tm. Reason behind non-amplification of other repeats may be either its absence or absence of motifs complementary to the primers in the genome.

The genetic diversity measured in terms of H, I, Ht, NPL and PPL among different valleys (i.e. Suru, Indus and Nubra valleys) found to be higher for Indus valley and least for Suru valley individuals indicating more variability in Indus valley and least in Suru valley individuals (Table 3.4).

3.3.4 ISSR derived dendrogram analysis

A dendrogram analysis using a total of 3,795 bands based on bootstrapping and neighbor joining (NJ) method grouped all the 90 genotypes into five main clusters (Figure 3.3). Cluster I contains individuals from Batalik (Suru) and Phyang (Indus) villages, cluster II represents individuals from Phey and Phyang (Indus). However, cluster III is a mixed cluster having majority of individuals from Nimmu, Basgo (Indus) and Skuru (Nubra), clusters IV and V have the individuals from Thiksey (Indus), Skampuk (Nubra) and Thiksey (Indus), Tirchey, Skampuk (Nubra) respctively. However, all the genotypes were clustered at populations level with bootstrap support value of > 75% (Figure 3.4a). An unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the populations suggested that the genotypes were more likely distributed (at high likelihood probability) with respect to their geographical distribution albeit small interference (Figure 3.4b).

Thus, our RAPD and ISSR data suggest that although an isolation-by-distance pattern may be detected across the whole range of *C. spinosa*, but the relationships between geographical and genetic distances have different patterns at different spatial scales. Similarly distinct patterns at different spatial scales were also found for some other plant species [200].

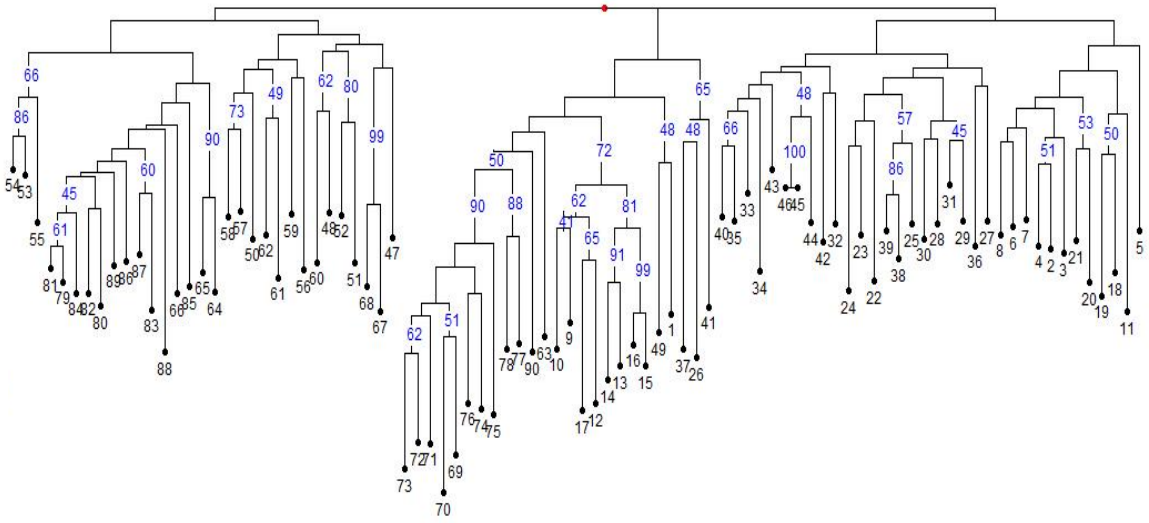


Figure 3.6 Dendrogram generated by NJ clustering technique showing relationships between 90 genotypes of *C. spinosa* collected from 9 different populations *viz.* Batalik (1-8); Nimmu (N912); Basgo (13-16); Phyang (17-31); Phey (32-46); Thiksey (47-62); Skampuk (63-68); Skuru (69-78); Tirchey (79-90) based on ISSR profiling. Number indicates bootstrap support values.

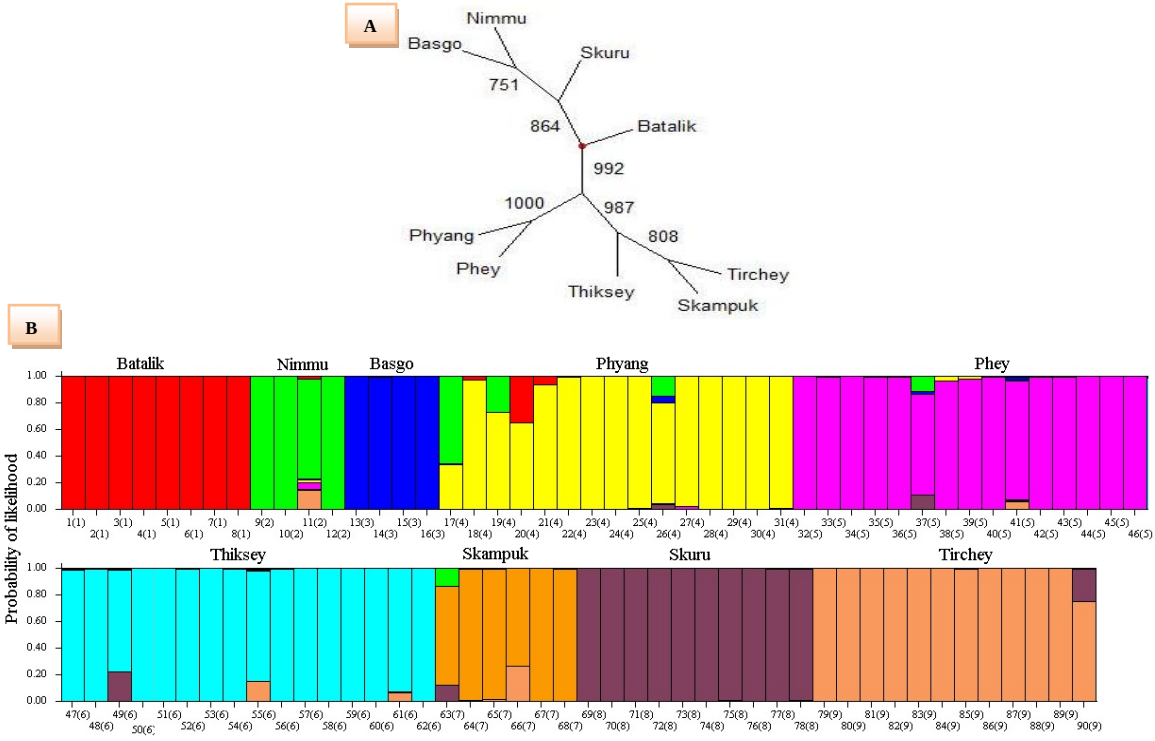


Figure 3.7 (A) NJ tree representing clustering of genotypes at populations' level along with supported bootstrap values, (B) Statistically unbiased clustering of 90 genotypes at population level. Genotypes from different populations are represented with different colours: Batalik (red), Nimmu (green), Basgo (blue), Phyang (yellow), Phey (pink), Thiksey (light blue), Skampuk (brown), Skuru (deep brown) and Tirchey (light brown).

3.3.5 Genetic variability details from RAPD+ISSR combined data

When H, I, Ht, Hs, NPL and PPL parameters were analyzed for valley divisions, then most of the respective values were found higher for Nubra valley individuals and least for Suru valley individuals (Table 3.4).

3.3.6 RAPD+ISSR combined data for cluster analysis

Dendrogram analysis using a combined data of 11742 RAPD and 3,795 ISSR bands based on bootstrapping and neighbor joining (NJ) method grouped all the 90 genotypes into six main clusters (Figure 3.3). The cluster I and II contains individuals from Tirchey (Nubra) and Thiksey (Indus) respectively. However cluster III and IV represents individuals from Skuru (Nubra) and Skampuk (Nubra), Thiksey (Indus) villages respectively. Phyang, Basgo, Nimmu (Indus) and Batalik (Suru) samples were clustered together in cluster V. However, cluster VI is having individuals from Phyang and Phey (Indus). However, all the genotypes were clustered at populations level with bootstrap support value of > 70% (Figure 3.4a). An unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the populations suggested that the genotypes were more likely distributed (at high likelihood probability) with respect to their geographical distribution albeit small interference (Figure 3.4b). Both RAPD and ISSR clusters showed partial similarity with combined data of RAPD+ISSR. Besides, principal component analysis (PCA) was also comparable to the cluster analysis (Fig. 3.10A). This is further confirmed through unbiased structuring of genotypes using STRUCTURE analysis at population level (Fig. 3.10B). In RAPD, ISSR and RAPD+ISSR the first three most informative PC components explained 31.87%, 37.59%, 30.99% of the total variations respectively.

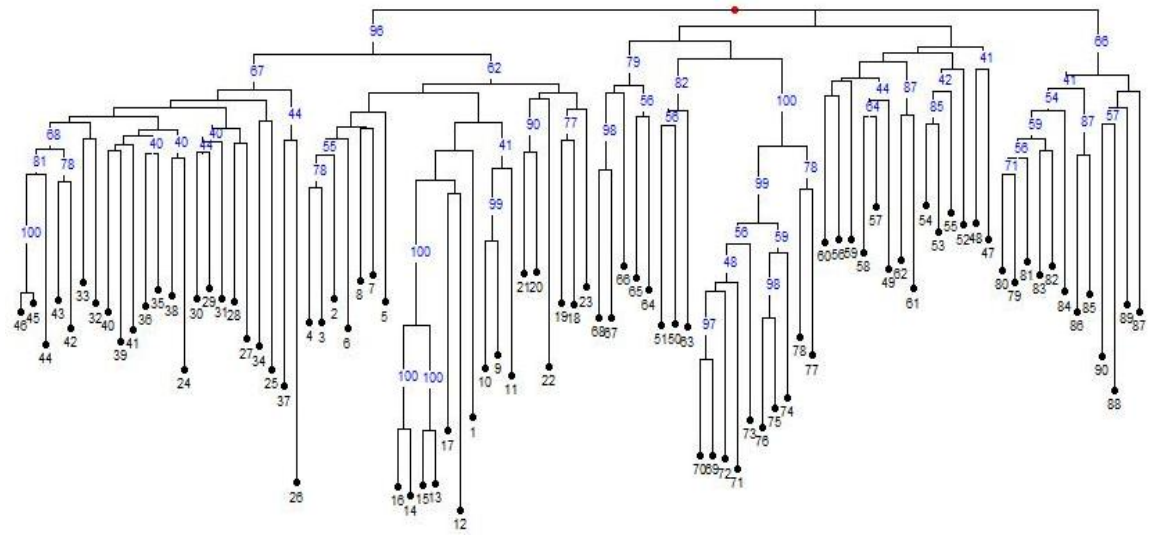


Figure 3.8 Dendrogram generated by NJ clustering technique showing relationships between 90 genotypes of *C. spinosa* collected from 9 different populations *viz.* Batalik (1-8); Nimmu (9-12); Basgo (13-16); Phyang (17-31); Phey (32-46); Thiksey (47-62); Skampuk (63-68); Skuru (69-78); Tirchey (79-90) based on RAPD+ISSR profiling. Number indicates bootstrap support values.

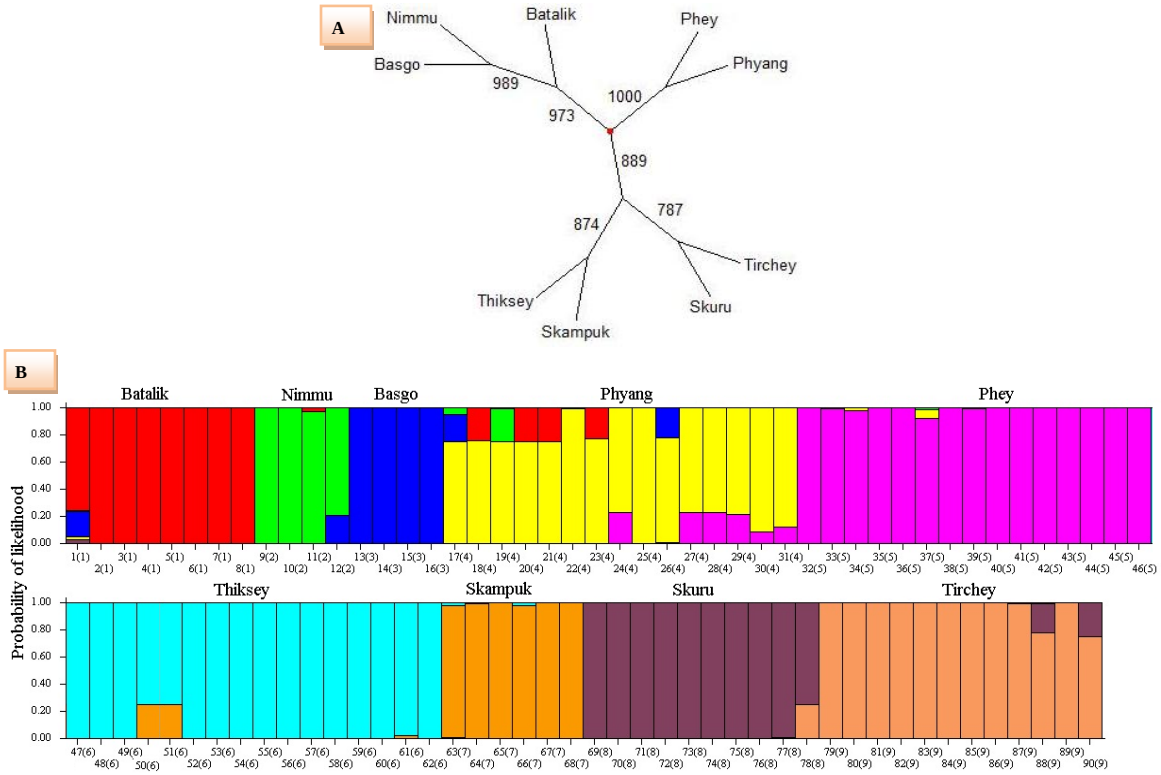


Figure 3.9 (A) NJ tree representing clustering of genotypes at populations' level along with supported bootstrap values, (B) Statistically unbiased clustering of 90 genotypes at population level. Genotypes from different populations are represented with different colours: Batalik (red), Nimmu (green), Basgo (blue), Phyang (yellow), Phey (pink), Thiksey (light blue), Skampuk (brown), Skuru (deep brown) and Tirchey (light brown).

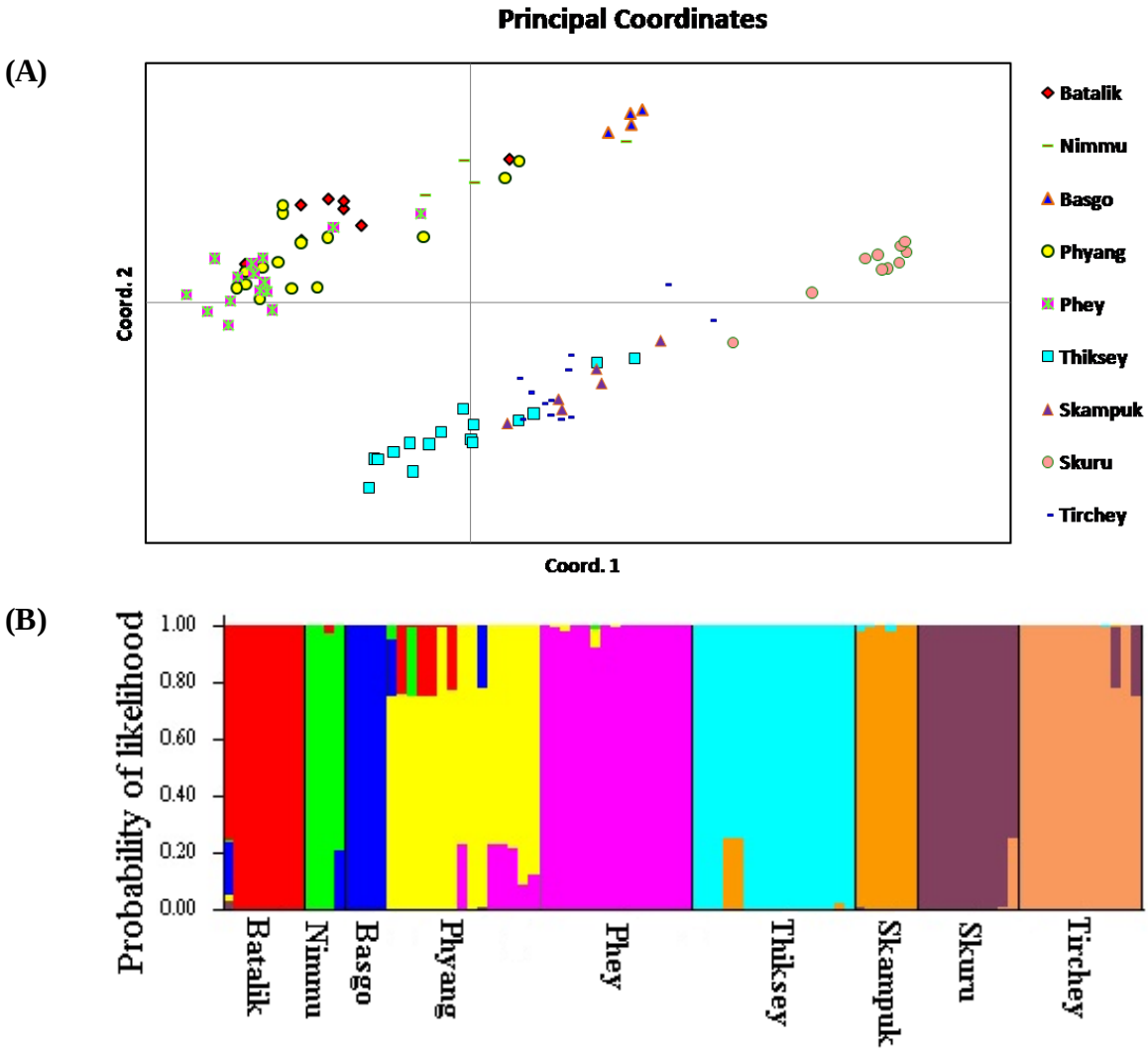


Figure 3.10 (a) Two-dimensional plot of principle component analysis of ninety *C. spinosa* individuals using RAPD+ISSR analysis. The shapes plotted represent individual cultivars. (b) Unbiased structuring of genotypes using STRUCTURE analysis at population level for RAPD+ISSR combined data.

A lower number of clusters are detected in all the three cases i.e. RAPD, ISSR and RAPD+ISSR than the number of populations sampled as also reported by Montes *et al.* [201]. Given that *C. spinosa* are long lived perennials and fragmentation has been more intense during the past 20-30 years, the expected effects of fragmentation on genetic diversity may take longer to express itself than in a shorter lived species. It is also possible that actual rate of out-crossing and gene flow are sufficient to maintain observed level of genetic variation within fragmented populations. Our results suggests that the

strong genetic structure of this species makes it potentially susceptible to variations in the mating system (inbreeding) and the effects of drift induced by reductions in population size and isolation.

The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (11,742 for RAPDs and 3,795 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships [199]. Dendrograms in the present study did not indicated clear pattern of clustering for within valley samples but, almost clear pattern was observed in all the three cases for between valley samples of Indus and Nubra valley. However, Suru valley genotypes showed similarity to Indus valley individuals and few individuals from Thiksey (Indus) genotypes were found more close to Nubra genotypes. The genetic closeness among the Indus valley and Suru valley cultivars can be explained by the high degree of commonness in their individuals which is same as observed in blackgram [202].

3.3.7 Comparative analysis of RAPD with ISSR markers

ISSR markers were found more efficient with regards to polymorphism detection, as they detected 100.0% as compared to 98.68% for RAPD markers. This is in agreement with the result of plant species like *Vigna* [203].

3.3.8 Diversity index (DI), Effective Multiplex Ratio (EMR) and Marker Index (MI)

DI, EMR and MI are used to measure and compare the informativeness of one marker with other *viz*. RAPD and ISSR [204, 205]. In the present investigation all these parameters were found high for ISSR than RAPD (Table 3.5) which highlights the distinctive nature of these markers. The higher number of fragments per primer combination in RAPD and ISSR provides higher EMR however Baghizadeh et al. [205] found that although SSR markers had the lowest values of the EMR and MI, but they had the highest level of polymorphism in pistachio cultivars. However, MI as a measure of

overall marker utility is applicable to any experimental situation where H and E may be calculated and hence MI may be used to predict the relative utilities of the various marker types for unknown germplasm [194]. Along with MI value, Prevost and Wilkinson [112] used resolving power (RP) to compare the informativeness of AFLP. Lack of correlation between EMR, MI and RP in our study, or lack of consistency in the correlation in other studies [204], makes it clear that probably a single parameter is not a good indicator to assess the informativeness of any primer.

Table 3.5 Summary of genetic variability across all the 90 individuals based on RAPD, ISSR and RAPD+ISSR analysis

Marker Type	Gst	Fst	DI	EMR	MI
RAPD	0.119	0.163	0.887	11.15	9.908
ISSR	0.033	0.192	0.972	10.625	10.325
RAPD+ISSR	0.032	0.136	-	-	-

Fst = Wright's inbreeding coefficient; DI = Diversity index; EMR = Effective Multiplex Ratio; MI = Marker Index.

3.3.9 Fst, Gst and AMOVA

The population differentiation (Fst) values from RAPD and ISSR is 0.163 and 0.192 ($P<0.001$) respectively (Table 3.5), which means 83.70%, 80.80% and 16.3%, 19.20% genetic variation is within the population and among populations respectively indicating that intra-population variation is more than the inter-population variations. Similarly Montes et al. [201] also reported that all Fst values were found significantly higher than expected by random structuring of *Heliconia* population.

The Gst value from RAPD, ISSR and RAPD+ISSR were 0.119, 0.033 and 0.032 respectively indicating that 88.1%, 96.7% and 96.8% of the genetic diversity resided within the population (Table 6). Molecular variance from RAPD, ISSR and RAPD+ISSR among valley (5.44, 7.14, 5.9%), among population (34.14, 32.21 and 33.61%) and among genotypes (60.41, 60.63 and 60.48%) (Table 3.6). It revealed that the genotypes

are genetically more diverse from each other. Besides this it again reconfirm the equal efficacy of both the marker for genetic diversity studies in *Capparis spinosa*. Thus, the expectation that genetic variability would decline due to population fragmentation was not supported in this case as observed by Hou and Lou [200]. This result might be observed because *C. spinosa* still occurs in medium or large population sizes (50 to over 100 individuals) in some localities. Another explanation could be that this high genetic diversity is a reflection of high historic genetic variability, which is quite common in long-lived perennial plant species [206].

Table 3.6 Summary of nested analysis of molecular variance (AMOVA) based on RAPD, ISSR individually and in combination, among the populations of *Capparis*. Levels of significance are based on 1000 iteration steps

Source of variation	**Among valleys**			**Among populations**			**Among genotypes**		
Markers	**RAPD**	**ISSR**	**RAPD + ISSR**	**RAPD**	**ISSR**	**RAPD + ISSR**	**RAPD**	**ISSR**	**RAPD + ISSR**
Variance component	2.450	1.211	3.661	15.377	5.459	20.745	27.227	10.275	37.503
Percentage	5.44	7.14	5.90	34.14	32.21	33.61	60.41	60.63	60.47
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

To further test this population structure, a model-based clustering method was implemented using program STRUCTURE. Without prior information about the populations and under an admixed model, STRUCTURE showed three as the likely number of populations for RAPD, ISSR and RAPD+ISSR data i.e., ΔK reached its maximum at K= 3 (Fig. 3.11a, b), suggesting that all populations fell into one of the three clusters. These three genetically distinct clusters primarily correspond to the geographic distribution of these populations (Table 3.1). The red cluster covered all populations from Suru valley, green cluster represent populations from Indus valley and the remaining populations were grouped in the blue cluster which is primarily consisting of genotypes from Nubra valley (Fig. 3.11a, b). The congruence between the geographical distribution

of populations and their genetic relationships is generally interpreted as sign of a long standing pattern of highly restricted gene flow [207].

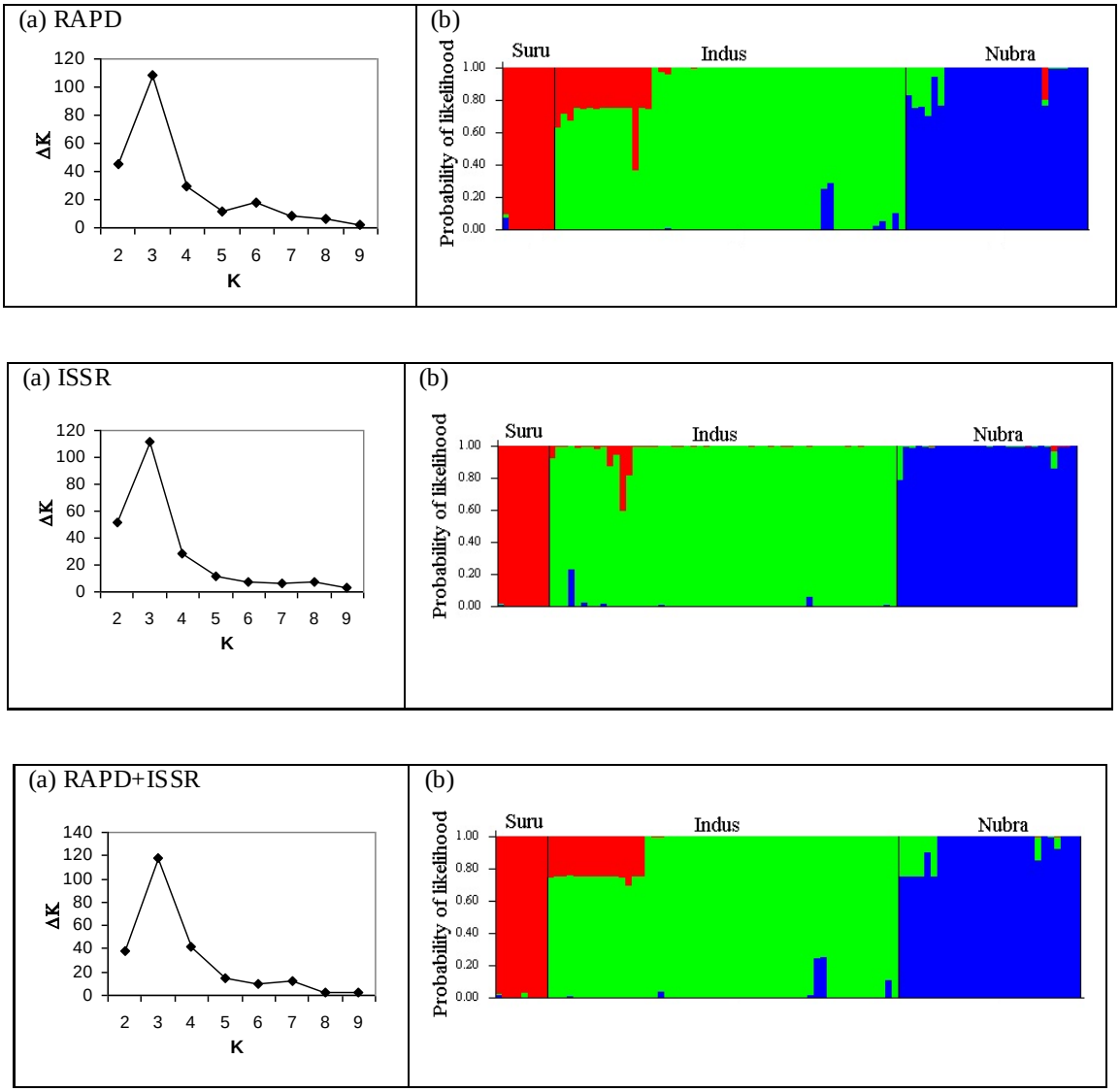


Figure 3.11 Analyses of STRUCTURE results for the determination of unique clusters with in the distribution range of *Capparis spinosa* (a) Average of ΔK for each K of independent runs, showing three as the likely number of populations for RAPD, ISSR and RAPD+ISSR data. (b) unbiased grouping when K=3.

In addition to demographic history, a great number of factors relative to life history and species biology *viz.* pollen and seed dispersal, successional stages, geographic distribution range, and mating systems can shape the levels and distribution of genetic

variability among and within populations [208]. In trans-Himalayas, at more than 3000 m above mean sea level, there are an array of factors which leads to deviation in partitioning of total genetic variation of a plant species, such as short vegetation period (about 120 days), wide temperature range (-40 to +35 ºC) and high UV 'B' radiations. Under such unique environmental conditions, no regular pattern of seed dispersal was observed in perennial plants like *C. spinosa*. Besides this germination rates are very low under natural conditions (2-4%), although under experimental conditions the rates are up to 62%. In this context, the genetic diversity within population is mostly depended on the first colonizing plants.

3.3.9 Conservation measures

Considering that all *C. spinosa* plants occur wild in trans-Himalayas with no conservation measures, we propose the establishment of new populations in private areas like farmers field and areas under governmental protection. As the plants of *C. spinosa* produce many fruits containing several seeds each, a strategy such as *ex-situ* preservation of seeds in seed bank is recommended. In this context, DIHAR has taken initiative to conserve the seeds of different population collected from various valleys of Ladakh under its permafrost based national germplasm conservation facility at Chang-La (5360 m AMSL). Considering the low diversity of Suru valley populations, we propose that saplings to be introduced in this valley population should preferably come from the same population in order to maintain the population distinctiveness. Another important measure is to carry out surveys to uncover more individuals and populations in other localities. Additionally, in some regions where possibly of habitat destruction or exploitation of plants especially fruits for local consumption are high, it is necessary to establish sustainable management plans and adequate collection of the fruits. Considering the critical situation of *C. spinosa*, probably the safest way to preserve the species is through all of the methods mentioned above. Furthermore, conducting new studies on the genetic health status of this species is required.

3.4 CONCLUSION

In conclusion, the total genetic diversity of *C. spinosa* was high, and both RAPD and ISSR markers were equally useful for studying the genetic relationships of *Capparis* individuals from the trans-Himalayan region of Ladakh. The geographical distribution of populations and their genetic relationships were quite consistent and most likely due to the natural geographic fragmentation of this species.

CHAPTER 4

ESTIMATION OF ANTIOXIDANT ACTIVITY AND TOTAL PHENOLICS AMONG NATURAL POPULATIONS OF CAPER (*CAPPARIS SPINOSA* L.) LEAVES COLLECTED FROM COLD ARID DESERT OF TRANS-HIMALAYAS

ABSTRACT

Antioxidant activity (%) of *Capparis spinosa* leaves collected from nine different sites from three valleys in trans-Himalayan region of Ladakh (India) were measured using DPPH, ABTS and FRAP assay along with its polyphenol content. Maximum DPPH and ABTS radical scavenging activity was observed from leaves samples collected from *Skuru* and least from *Tirchey* site. FRAP assay revealed that *Skuru* site possess maximum antioxidant content as compared to the samples collected from any other location. IC_{50} of ABTS were quite reasonably correlated with FRAP assay (R2=0.517) while, DPPH IC_{50} was poorly correlated with both ABTS (R^2=0.100) and FRAP assay (R^2=0.223). The highest and lowest phenolic and flavonoid content was recorded in *Skuru* and *Tirchey* sites respectively. Total phenolics (27.62-21.42 mg GAE/g DW) and flavonoid content (6.96-2.69 mg quercetin equivalent/g DW) were found reasonably correlated with IC_{50} of ABTS (R^2=0.741 and 0.703, respectively) and FRAP (R^2=0.605 and 0.649, respectively) but poorly correlated with DPPH IC_{50} (R^2=0.303 and 0.407, respectively). Results of present study confirmed the antioxidant potential of *C. spinosa* leaves collected from different locations of trans-Himalayas, whose phyto-chemistry and phyto-pharmacology should be investigated further in order to detect possible phyto-therapeutic uses where free radicals are implicated.

4.1 INTRODUCTION

The wild edible plants are rich source of phyto-chemicals, such as carotenoids, flavonoids and other phenolic compounds having high free-radical scavenging activity, which helps to reduce the risk of chronic diseases, such as cardiovascular disease, cancer, and age related neuronal degeneration [209]. *Capparis spinosa* (Capparaceae) – also called 'Caper' and locally known as '*Kabra'* – is an under-utilized wild plant, occasionally used by local people of trans-Himalayan region of Ladakh (India) as a leafy vegetable. In Ladakh, several types of medicinal preparations from *Capparis* are being used by the Amchis (local traditional doctors) for the treatment of various ailments like gastrointestinal infection, diarrhoea and rheumatism [36]. *Capparis* is known to contain a wide variety of antioxidant compounds including phenolic compounds which are found to be well correlated with antioxidant potential. Phenolics or polyphenols have received considerable attention because of their physiological functions, including antioxidant, anti-mutagenic and anti-tumor activities. Previous chemical studies on *C. spinosa* have shown the presence of alkaloids, lipids, polyphenols, flavonoids, indole and aliphatic glucosinolates [210].

Ethanolic extract from the fruit of *C. spinosa* exhibits a notable activity in protecting against oxidative stress and suggesting its protective effect against skin sclerosis [163]. However, methanolic extract of *C. spinosa* buds, rich in flavonoids, including several quercetin and kaempferol glycosides, was demonstrated to possess strong antioxidant/free radical scavenging effectiveness, antiviral and immunomodulatory effects in different *In vitro* tests [153]; *In vivo* this extract showed a noteworthy anti-allergic effectiveness against bronchospasm in guinea pigs [152], and when topically applied it afforded significant *in vivo* protection against UV-B light induced skin erythema in humans [23]. Natural antioxidants present in *Capparis* can scavenge harmful free radicals from our body and it is possible to reduce the risk of chronic diseases by either enhancing the body's natural antioxidant defences or by supplementing with proven dietary antioxidants [211].

The most widely used synthetic antioxidants in food (butylated hydroxytoluene BHT, butylated hydroxyanisole BHA) are very effective as antioxidants but their use in food products has been failing off due to their instability, as well as due to a suspected action as promoters of carcinogenesis. For this reason, there is a growing interest in the studies of natural healthy (non-toxic) additives as potential antioxidants [212]. Hence, focusing our attention on natural sources of antioxidants, this study was carried out to evaluate the antioxidant activity and total polyphenolic content of *C. spinosa* tender leaves collected from nine different locations from three valleys of trans-Himalayas of Ladakh region (India). Moreover, the antioxidative potential of tender caper leaves was less known from any part of the world. In additions, the correlation between antioxidant capacity and polyphenol content was also determined. Besides nutritional qualities, data on antioxidant capacity and polyphenol content would be additional information to be considered when promoting the consumption of caper in different forms.

4.2 MATERIALS AND METHODS

4.2.1 Plant collection

Fresh tender leaves used in the present investigation were collected from *Capparis* plants growing in wild from three valleys and nine villages of Ladakh during the month of July 2009 (Table 4.1). Ladakh is a part of Indian Himalaya at an altitude of 2200-6100 m above mean sea level, is characterized by diverse and complex land formations. It is located at the latitude of 31º44'57"–32º59'57"N and longitude of 76º46'29"–77º41'34"E. The extreme low temperature during winter months (-40 °C), low annual precipitation (20–30 mm) along with low relative humidity (20-40%) make this region a typical cold arid dessert. The plant was identified by its vernacular name by the local people and later authenticated at the Herbarium of Defence Institute of High Altitude Research, Leh-Ladakh, India.

4.2.2 Chemicals

2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS+), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium ferricyanide,

potassium persulfate, trichloroacetic acid, gallic acid and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), FeCl3, ascorbic acid, and butylated hydroxyl toluene (BHT) from HIMEDIA Laboratories Pvt. Ltd. (Germany); Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate (Na2CO3), hydrochloric acid (HCl), glacial acetic acid, potassium chloride, sodium acetate trihydrate and solvent methanol were obtained from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used, were of analytical grade.

Table 4.1 Collection sites of *Capparis* leaves (along with altitude) from the trans Himalayas of Ladakh valley.

Valleys	Sampling sites	Altitude (m)
Suru	Batalik	3310
Indus	Basgo	3320
	Nimmu	3242
	Phyang	3347
	Phey	3175
	Thiksey	3435
Nubra	Skampuk	3197
	Skuru	3117
	Tirchey	3159

4.2.3 Determination of antioxidant activity

4.2.3.1 Extract preparation

Leaves were air dried in shade at room temperature (26 °C) to constant weights. The dried plant materials were ground separately to powder. Ten grams of each ground plant materials were shaken separately in methanol for 72 hrs on an orbital shaker at room temperature. Extracts were filtered using a Buckner funnel and Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressures at 40 ºC using a rotary evaporator. Each extract was resuspended in methanol to make 50 mg/ml stock solution.

4.2.3.2 DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana- Pathiranan and Shahidi [213]. The absorbance of the mixture was measured

spectrophotometrically at 517 nm using BHT as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs Control – Abs Sample)]/(Abs Control)]x100 where; Abs Control is the absorbance of DPPH radical+methanol; Abs Sample is the absorbance of DPPH radical+sample extract / standard.

4.2.3.3 ABTS radical scavenging assay

ABTS radical scavenging assay was determined according to method of Re et al. [214]. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity (%) = [(Abs Control – Abs Sample)]/(Abs Control)]x100 where; Abs Control is the absorbance of ABTS radical+methanol; Abs Sample is the absorbance of ABTS radical+sample extract/standard. IC_{50} (Inhibition coefficient) value was determined from the plotted graph of scavenging activity against the concentration of leaf extract collected from different sites, which is defined as the amount of antioxidant necessary to decrease the initial DPPH/ABTS radical concentration by 50%.

4.2.3.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted using method of Wong et al. [215]. The increase in absorbance was measured using spectrophotometer at 593 nm. The percent of antioxidant was calculated using the formula, percent of antioxidant (%) = [(Abs Sample – Abs Control)/ Abs Sample] x 100.

4.2.3.5 Determination of total phenol contents

Total phenol content in the extracts was determined by using modified Folin-Ciocalteau method [216]. Absorbance of the solution was measured at 765 nm. Total phenolic content was expressed as mg/g tannic acid equivalent using the following

equation based on the calibration curve: y = 0.1216x (R^2=0.9365), where x was the absorbance and y was the Gallic acid equivalent (mg/g).

4.2.3.6 Determination of total flavonoid contents

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon et al. [217]. The absorbance of the solution was measured at 420 nm and yellow color indicated the presence of flavonoids. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.0255x (R^2=0.9712), where x was the absorbance and y was the quercetin equivalent (mg/g).

4.2.4 Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates (*n*=3). Where applicable, the data were subjected to one way analysis of variance (ANOVA) using SPSS 11.5. Duncan's multiple range tests was used to assess difference between means. Pearson's correlation test was used to assess correlation between means. Dendrogram was constructed using Ward method and distance is expressed as Euclid distance. *P-v*alues < 0.05 were regarded as significant.

4.3 RESULT AND DISCUSSION

4.3.1 Radical scavenging activity by DPPH and ABTS

The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. Fig. 4.1 shows the IC_{50} values of DPPH and ABTS radical scavenging activity of the methanolic extracts of *C. spinosa* leaves, collected from 9 sites from trans-Himalayan region of Ladakh. The DPPH test provides information about the activity of test compounds with stable free radicals and its effect is thought to be due to their hydrogen donating ability. For the DPPH radical, Skuru samples showed lowest DPPH based IC_{50} (0.066 mg/ml) while Tirchey (0.077 mg/ml) had highest IC_{50} value (Fig. 4.1). The samples from other location like Basgo = Thicksey = Phey = Skampuk and Nimmu = Phyang = Tirchey were not significantly different from

each other ($p>0.05$). Higher the IC_{50} value signifies less antioxidant activity and *vice-versa*. This concludes that Skuru sample have maximum antioxidant activity while, samples collected form Nimmu, Phyang and Trichey have least antioxidant activity. The average IC_{50} values for DPPH radicals obtained were 0.073 mg/ml.

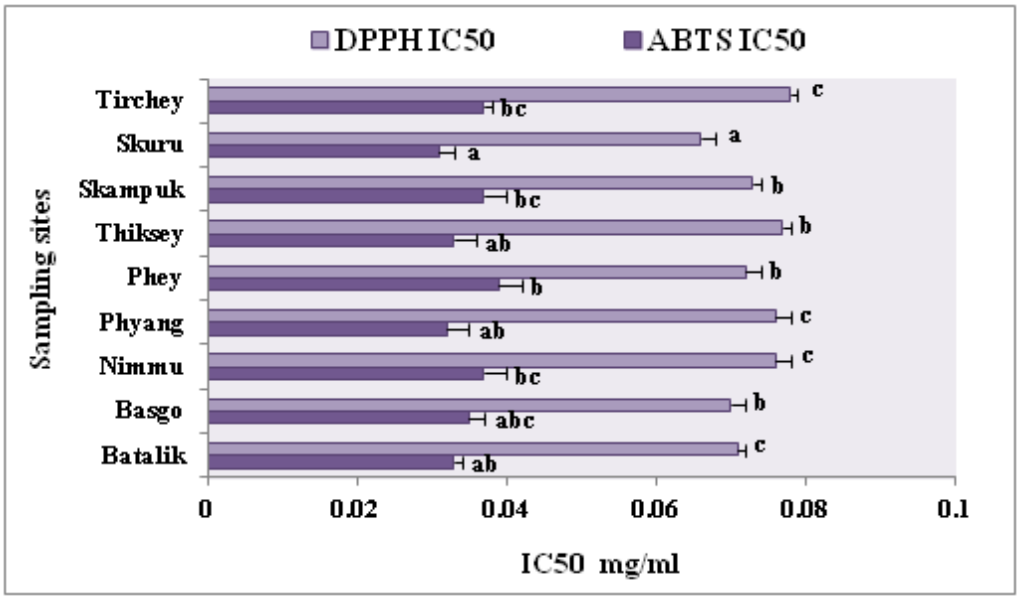


Figure 4.1 IC_{50} value for DPPH assay (faint purple bars, mg/ml) and ABTS assay (dark purple bars, mg/ml) of *C. spinosa* leaves collected from different sites in Ladakh region.

For the ABTS radical, leaves collected from Skuru showed lowest IC_{50} value (0.031 mg/ml) and samples collected from Phey showed highest IC_{50} (0.039 mg/ml) value. The IC_{50} values of other samples were given in the Fig. 4.1. This showed that Skuru sample have maximum antioxidant activity while, samples collected form Nimmu, Skampuk, Trichey and Phey had least antioxidant activity. The average IC_{50} values for ABTS radicals obtained were 0.034 mg/ml. Apparently, the antioxidant activities of other samples showed almost similar trend as observed with DPPH method.

The DPPH activity of all the *Capparis* samples studied were significantly lower than their ABTS values (Fig. 4.1). Average IC_{50} values determined by ABTS assay were almost twice lower as compared to values determined by DPPH assay. Awika *et al.* [218] reported minimal difference between DPPH and ABTS values of brown sorghum samples. However, for the black sorghum, the authors observed that all of the ABTS

values were significantly higher than the DPPH values. Teow *et al.* [219] also observed similar results in case of sweet potato. Factors like stereo-selectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals [220]. Wang *et al.* [221] found that some compounds which have ABTS scavenging activity did not show DPPH scavenging activity. This is not the case in this study. This further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

4.3.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and produce a coloured complex of ferrous tripyridyltriazine (Fe^{2+}–TPTZ) [222]. The FRAP assay is widely used in the evaluation of the antioxidant component in the dietary polyphenols [223]. Highest total antioxidant content (%) was observed in the leaves samples collected from Skuru (73.54-77.14%) while, lowest was found in Phey (47.97-73.37%) and Tirchey (53.26-74.07) samples (Fig. 4.2). However, not significant difference was observed for total antioxidant content (%) among other location samples. For BHT (positive control) this value varied between 37.50 to 75.97%.

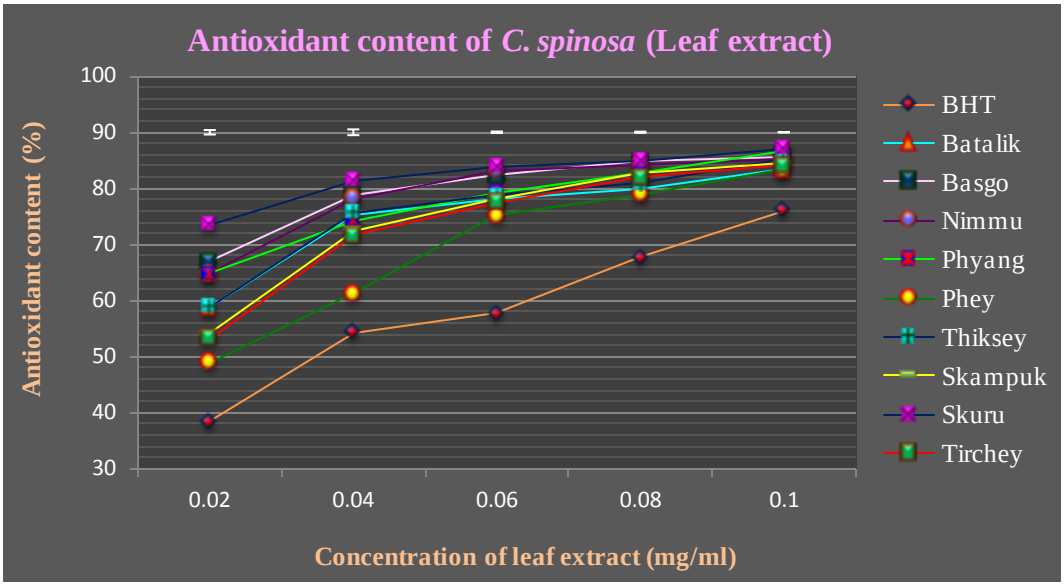


Figure 4.2 Antioxidant content (%) of *C. spinosa* leaves using FRAP assay.

Likewise in DPPH and ABTS assay, FRAP also showed lowest antioxidant activity (AOA) for Phey and Tirchey while highest for Skuru samples, suggesting that these methods have similar predictive capacity for AOA in *Capparis*. Antioxidant content (%) determined by FRAP assay ranged from 73.43 to 77.14% at 0.1 mg/ml of leaf extract and increase in AOA was almost similar in DPPH and ABTS assay (Fig.4.2).

4.3.3 Total phenols

Phenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [224]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [225]. Phenolic content in the *Capparis* leaves as determined by the Folin-Ciocalteu method ranged from 21.42 (Tirchey) to 27.62 mg GAE/g DW (Skuru). The samples collected from Nimmu, Phey, Skampuk and Thicksey, Basgo and Batalik, Phyang were not significantly different ($p<0.05$) from each other (Fig. 4.3). This correlate with the finding that Skuru sample have maximum antioxidant activity while samples collected form Tirchey have least antioxidant activity which was observed with both DPPH and ABTS radical scavenging activity. Several studies showed a correlation

between antioxidant activity and phenolic content [226]. Genetic factors and growing conditions may play an important role in the formation of secondary metabolites, including phenolic acids [227]. According to Oktay *et al*. [228], a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species.

Based on the antioxidant assays, it is thus suggested that phenolic compounds present in the leaves of *Capparis* have strong scavenging ability and ferric reducing power. This could be due to the antioxidant activity of phenolic compounds towards free radicals. Beside phenolic compounds, the presence of flavonoids might also influence the antioxidant capacity. This result strongly suggests that polyphenol are important components of *Capparis*, which are responsible for not only its antioxidant activities but some of its pharmacological effects could be attributed to the presence of these valuable constituents.

4.3.4 Total flavonoids

The flavonoid content of *C. spinosa* leaves was shown in Fig. 4.3. The total flavonoid contents in these samples ranged from 2.69 to 6.96 mg quercetin/g dry wt. The result reveals that samples from Skuru having maximum flavonoids content (6.96 mg quercetin/g DW) while, Tirchey samples (2.6 mg quercetin equivalent/g DW) having minimum flavonoid content than the samples from any other location.

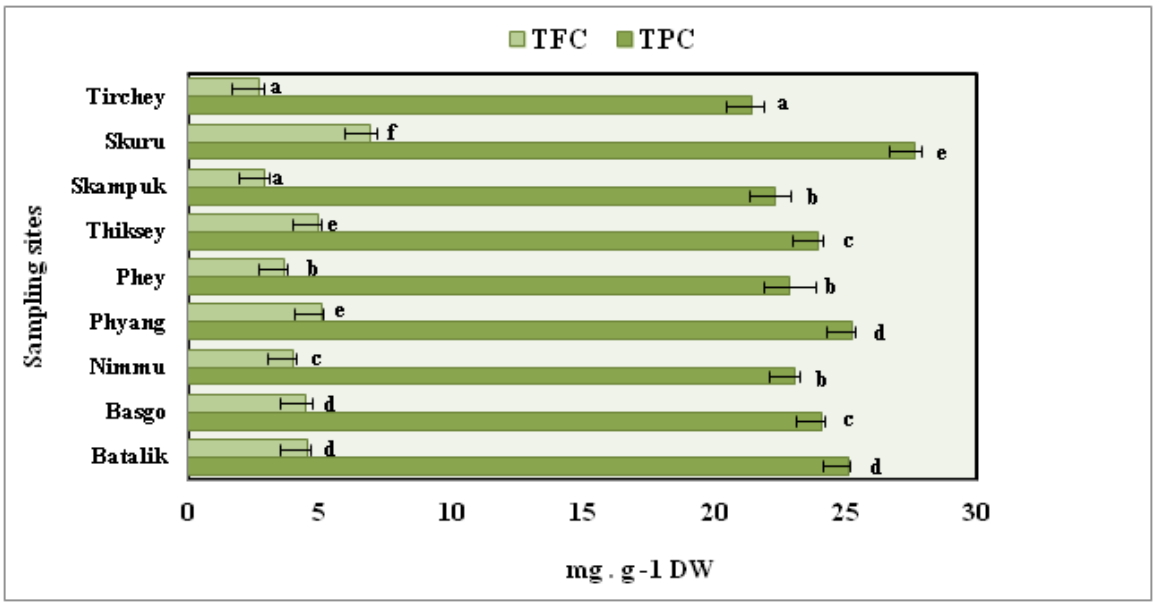


Figure 4.3 Total phenol content (mg of GAE g $^{-1}$ of DW) and total flavonoid content (mg of quercetin g $^{-1}$ of DW) of *C. spinosa* tender leaves.

4.3.5 Relationship between total phenolic content and antioxidant activity

From Fig. 4.4 it is apparent that contrary to ABTS and FRAP assay DPPH assay analyzed lesser response and sensitiveness, even if the results measured with the individual assay showed the same trend in the whole data set. Congruency of the obtained results determined by the individual methods was tested by linear regression and correlation of measured values between ABTS and FRAP assay was R^2=0.517 (Fig not shown), whereas between DPPH and ABTS assay and DPPH and FRAP assay it was significantly less (R^2=0.100 and R^2=0.223, respectively). These results show some congruency of ABTS and FRAP assay and conversely to their incompatibility to DPPH assay. Both DPPH and ABTS procedures are relatively inexpensive and simple to perform. The ABTS method can be used over a wide pH range [229], whereas the DPPH method is limited to neutral and higher pH applications. Additionally, the ABTS assay is much faster than the DPPH assay and not affected by colour interference.

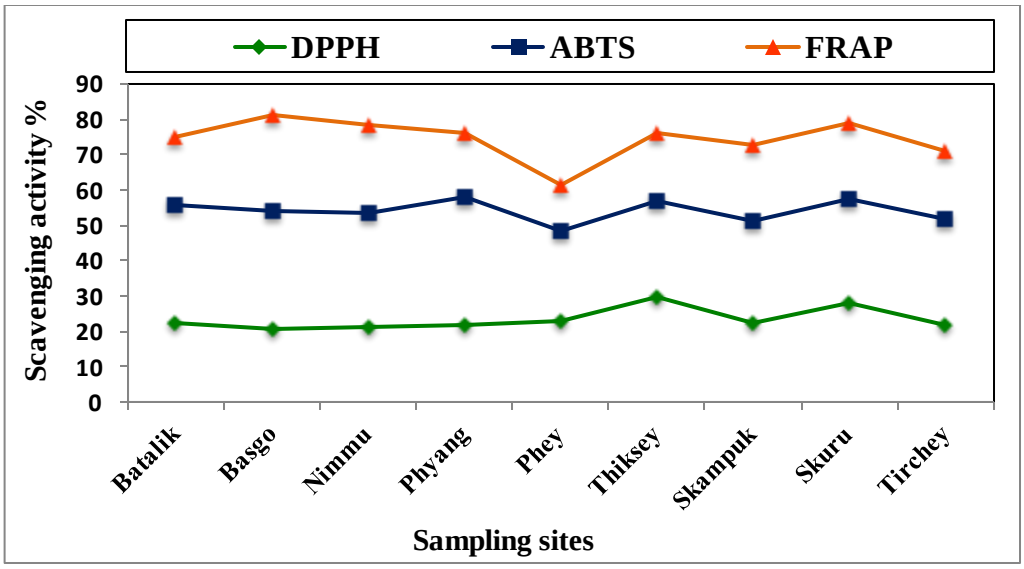
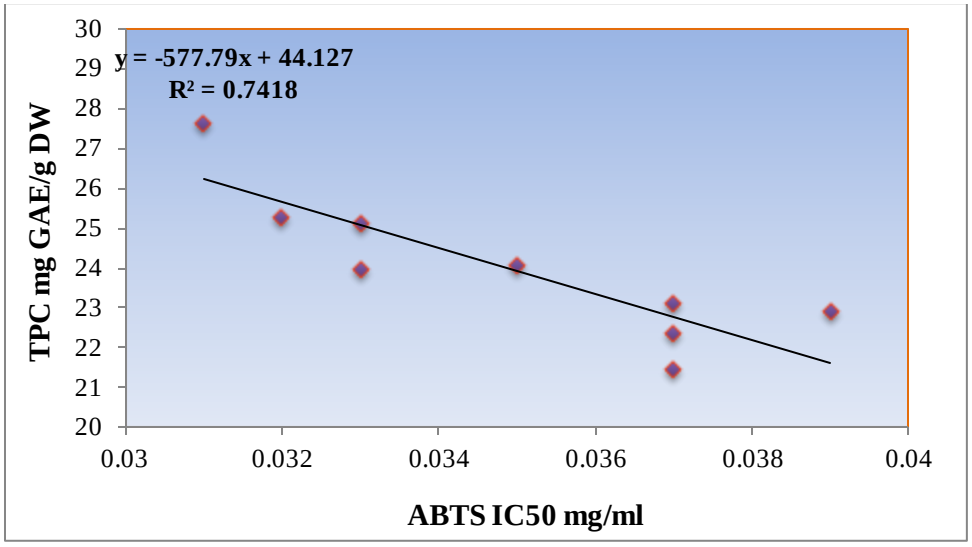


Figure 4.4 Free radicals scavenging activity determined with ABTS, DPPH and FRAP assays at 0.04 mg/ml concentration of methanolic extract of leaves of *C. spinosa* samples.

The results suggested that all the three methods have almost similar predictive capacity for antioxidant activities for *C. spinosa* leaves. However, the correlation of ABTS IC_{50} with DPPH IC_{50} value and FRAP assay activities for *C. spinosa* leaves collected from different locations in Ladakh region were relatively lower than those found for other food commodities. Leong and Shui [230] reported a high correlation (R^2=0.90) between ABTS and DPPH values for various fruit extracts. While, Lachman *et al.* [231] observer very high correlation (R^2=0.937) of measured value between ABTS and FRAP assay for honey samples.

Correlation between TPC and ABTS assay and TFC and ABTS assay was found as R^2=0.742 and R^2=0.703, respectively (Fig. 4.5a, b). While, correlation between TPC and FRAP assay and TFC and FRAP assay was at the level of R^2=0.605 and R^2=0.649, respectively (Fig. 4.6a, b). This result was in agreement with Othman *et al.* [232] who found a strong correlation between total phenolic content and FRAP assay. However, correlation between DPPH IC_{50} value and total phenolic and flavonoid content were not high as R^2=0.303 and R^2=0.407, respectively (Fig. 4.7a, b). Islam *et al.* [227] reported a relatively lower, but significant correlation coefficient (R^2=0.37) between DPPH activities and the total phenolic content of sweet potato. High correlation coefficients

between the phenolic content and antioxidant activities have been reported for various food commodities such as sorghum (R^2=0.971) [233] and cactus pear (R^2=0.970) [234]. Therefore, total phenolic content can be used as an indicator in assessing the antioxidant activity of fruits and vegetables. Prior *et al.* [237] recommended that the Folin-Ciocalteu method for total phenolic determination can be standardized for comparison of the result between laboratories.



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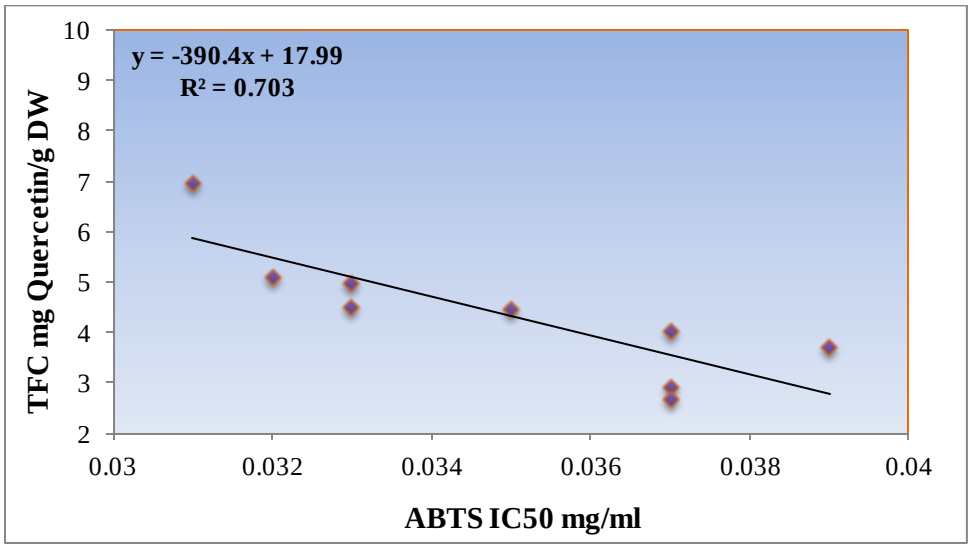
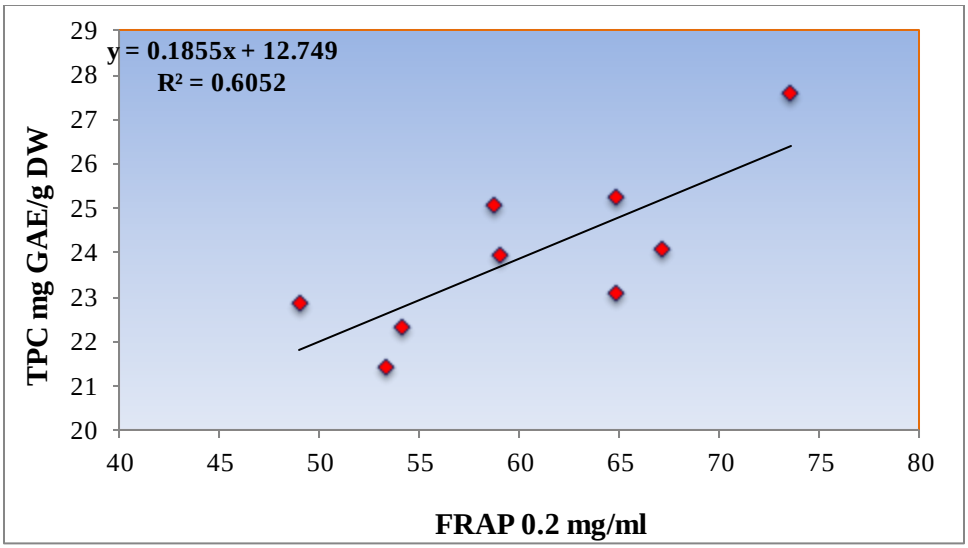


(b)

Figure 4.5 Linear correlations between **(a)** ABTS IC_{50} and TPC **(b)** ABTS IC_{50} and TFC



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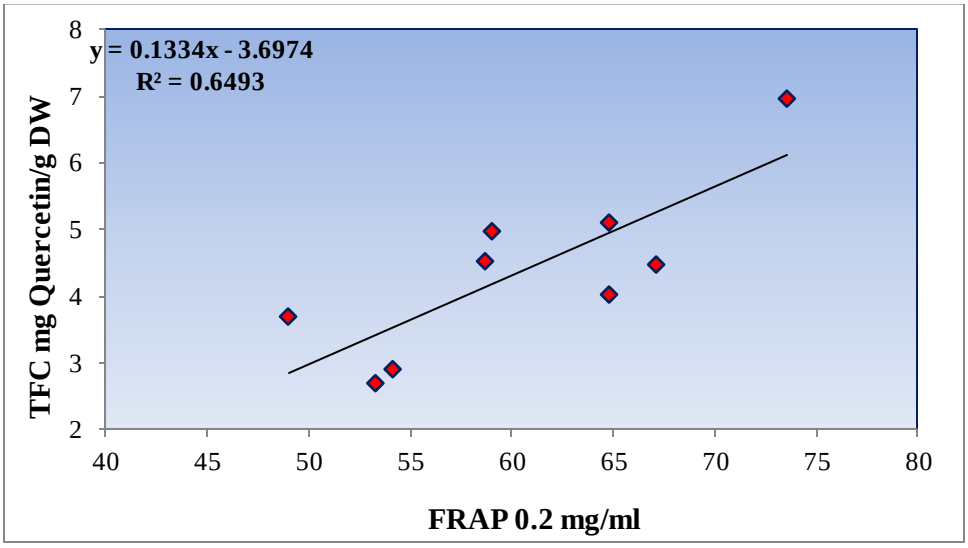
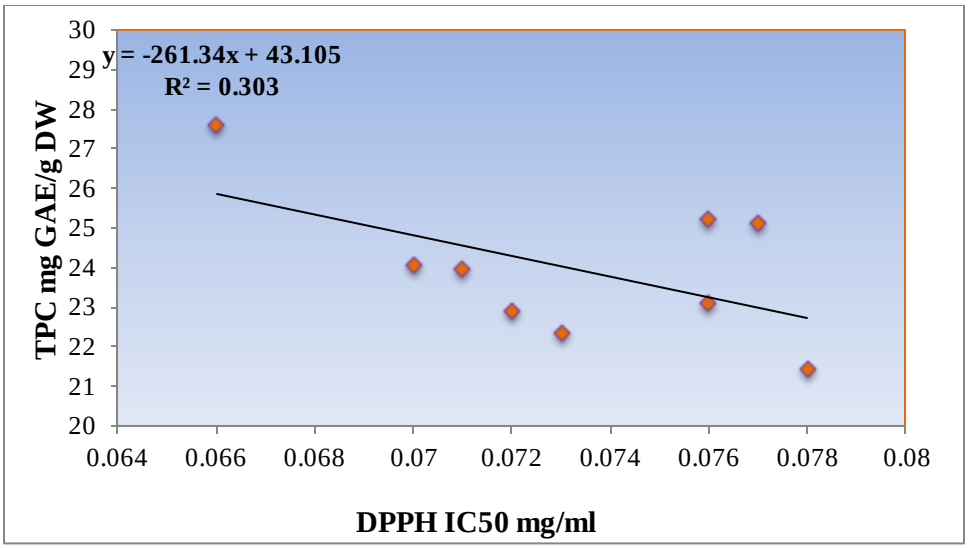


(b)

Figure 4.6 Linear correlation between **(a)** FRAP assay (0.02 mg/ml) and TPC **(b)** FRAP assay (0.02 mg/ml) and TFC.

The high degree of correlation between the simple spectrophotometric assay for total phenolic compounds and antioxidant activity of the methanolic extracts, as determined by radical quenching assays, shows that the assay for total phenolics would be a useful technique for rapid evaluation of antioxidant activity in *Capparis* leaves. This would be important for breeding programmes where many samples must be evaluated or for antioxidant activity between genotypes.



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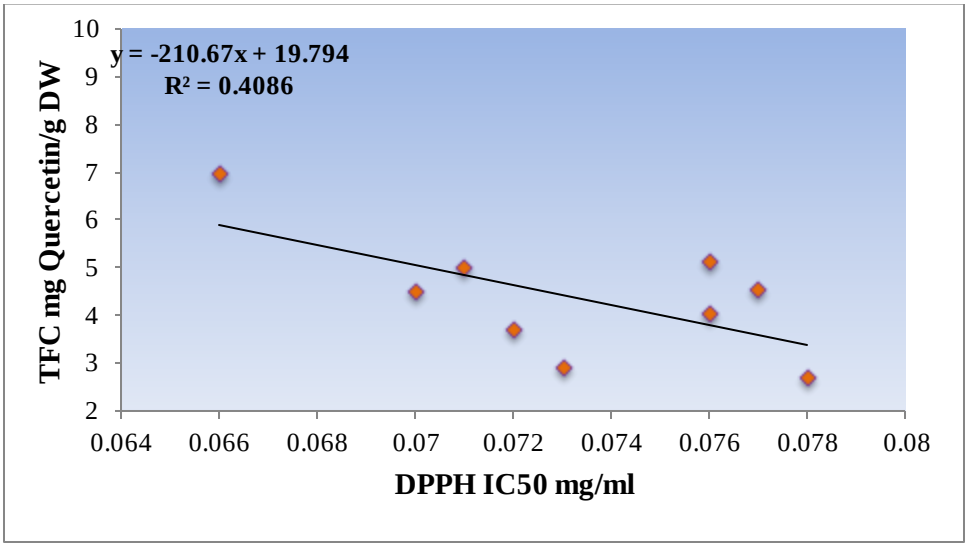


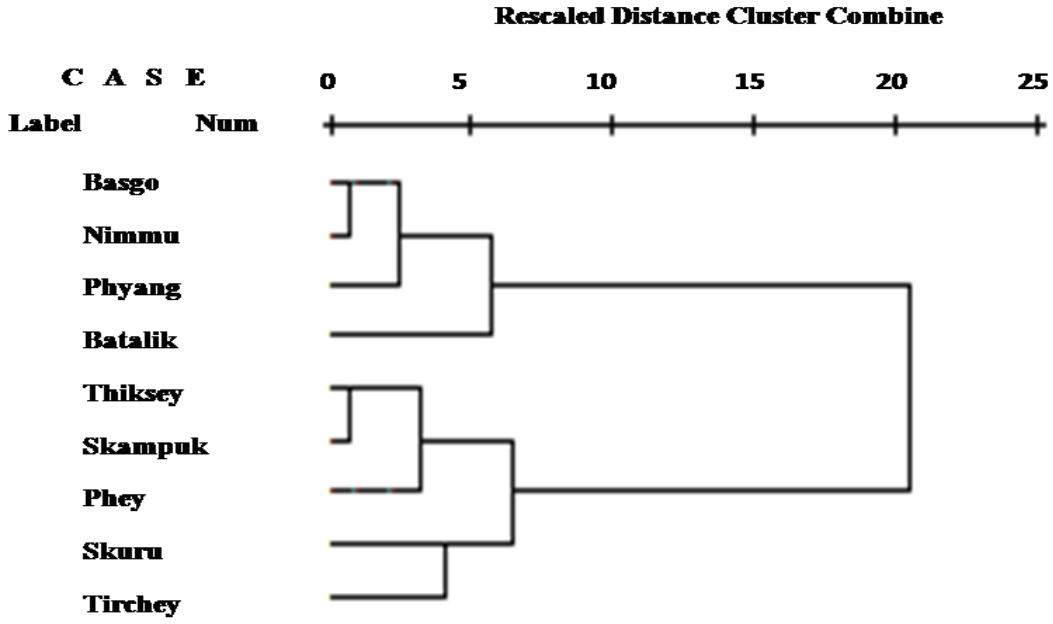
(b)

Figure 4.7 Linear correlations between **(a)** DPPH IC_{50} and TPC **(b)** DPPH IC_{50} and TFC.

4.3.6 Cluster Analysis

Cluster analysis of *C. spinosa* leaves collected from different sites in Ladakh region showed that cluster based on total antioxidant activity (DPPH, ABTS and FRAP) was almost similar to cluster based on total phenolic content. Where Basgo, Nimmu, Phyang, Batalik samples had fallen in one cluster while samples from Thiksey, Skampuk, Phey, Skuru and Tirchey had fallen in another cluster (Fig 4.8a and 4.8b). Whereas, in

case of cluster based on total flavonoid content samples from Phey and Thiksey are falling in different cluster (Fig 4.8c). It suggests that in case of capers, total AOA activity is more contributed by total phenolics than total flavonoids.



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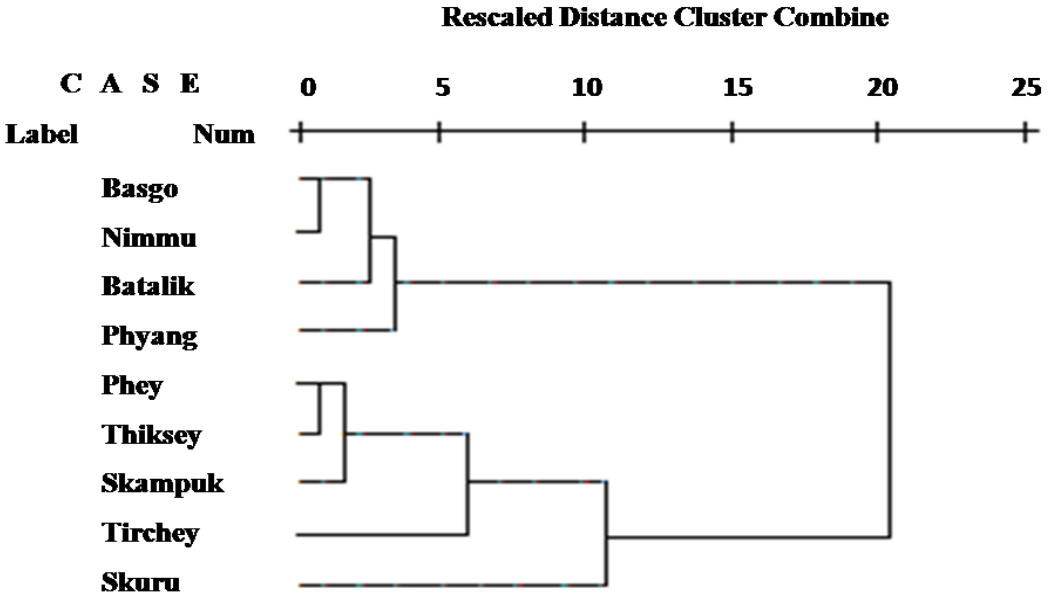

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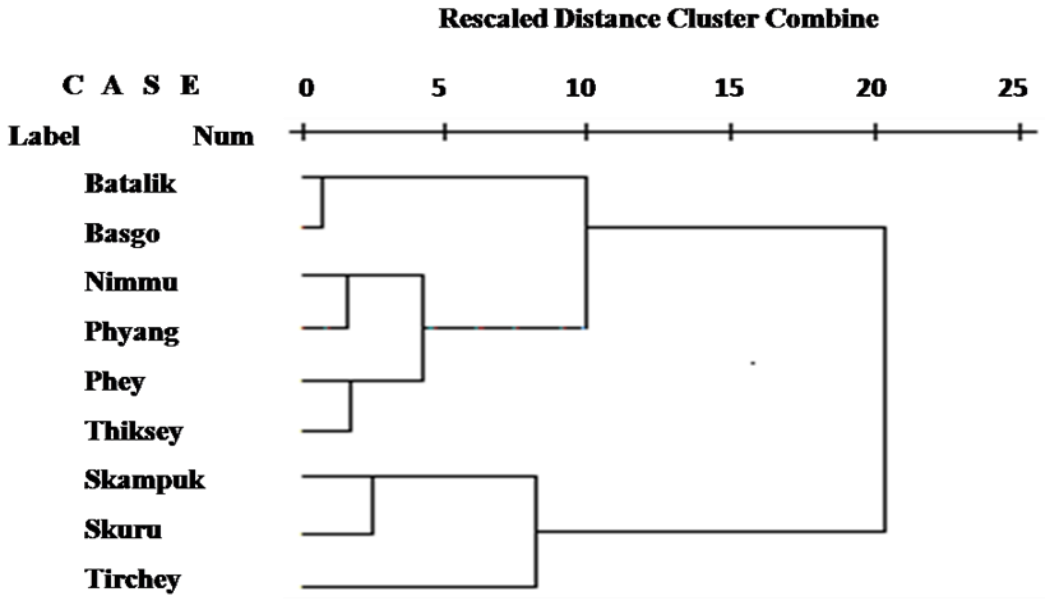


Figure 4.8 Dendrogram of different sampling sites according to cluster analysis of similarity using Ward method on the basis of **(a)**Total AOA determined by DPPH, ABTS and FRAP assays **(b)** Total Phenolic content **(c)** Total Flavonoid content where, distance is Euclid distance

4.4 CONCLUSIONS

Analysis of antioxidant activity by DPPH and ABTS method of methanolic extract of *C. spinosa* leaves collected from different sites in Ladakh region showed differences in antioxidant properties in individual samples of leaves. The highest values of antioxidant activity showed unambiguously in the leaves collected from Skuru and conversely the lowest values demonstrated in leaves collected from Tirchey and Phey. FRAP method revealed that samples from Skuru possess good antioxidant content comparable with that of the leaves collected from other localities. The highest total phenolic and total flavonoid content exhibits in the leaves collected from Skuru and lowest in the leaves collected from Tirchey which is same as observed for antioxidant activity. This result again suggests that antioxidant activity of capers is caused mainly by phenolics and flavonoids. By comparison of three antioxidant activity assays–ABTS, FRAP and DPPH–with different ability of used stable radicals to react with antioxidants, both ABTS and FRAP assay have been proved as a good parameter for the determination of antioxidant quality

of *C. spinosa* leaves. Thus, *C. spinosa* tender leaves may therefore be a good candidate for functional foods as well as plant-based pharmaceutical products.

CHAPTER 5

EVALUATION OF ANTIOXIDANT ACTIVITIES AND TOTAL POLYPHENOLS OF EDIBLE PARTS OF *CAPPARIS SPINOSA* L. COLLECTED FROM TRANS-HIMALAYA

ABSTRACT

The antioxidant activity and total polyphenols of the methanolic extract of leaves, flower buds, roots and fruits of *C. spinosa* were assessed in an effort to corroborate the medicinal and culinary potential of the edible parts of the plant. To estimate the mentioned antioxidant capacity, three different methods were performed: the 2,2-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity and ferric reducing/antioxidant power assay (FRAP). Highest DPPH and ABTS radical scavenging activity was observed in leaves and least in dried fruit samples. FRAP assay illustrated that leaves samples possess maximum antioxidant contents and dried fruit sample restrain minimum as compared to the other edible parts and well-known antioxidant butylated hydroxytoluene (BHT). The IC_{50} value of DPPH were highly correlated with IC_{50} value of ABTS (R^2=0.9074) and FRAP assay (R^2=0.9771). However, IC_{50} value of ABTS reasonably correlated with FRAP assay (R^2=0.5737). The highest phenolic and flavonoid content was found in leaves samples (24.77-5.69 mg GAE/g DW) and lowest in dried fruit samples (4.07-0.00 mg quercetin equivalent/g DW). The total phenolic contents were highly correlated with IC_{50} value of ABTS (R^2=0.9074), DPPH (R^2=0.9377) and FRAP value (R^2=0.9617). But total flavonoid contents were reasonably correlated with ABTS (R^2=0.7449), DPPH (R^2=0.7791) and FRAP value (R^2=0.9577). This study, has to some extent, validated the medicinal potential of all the edible parts of the *C. spinosa*.

5.1 INTRODUCTION

There is a growing demand for natural products in the human diet, both due to the possible negative effects of synthetic food additives on human health and to the increased consumer perception of this problem in recent years. Antioxidant components are microconstituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation of oxidizing chain reactions, and also involved in scavenging free radicals. A great number of edible medicinal plants contain chemical compounds that exhibit antioxidant properties. Edible parts of plant such as fruits, buds and roots are reported to contain a wide variety of antioxidant components, including phenolic compounds. These compounds are found to be correlated with antioxidant potential [235].

Capparis spinosa L. (Capparidaceae) also called 'Caper' and locally known as '*Kabra'* is one of the oldest known medicinal plant in 'Amchi system' (local medicinal system) for the treatment of various ailments like gastrointestinal infection, diarrhoea and rheumatism and occasionally used by local people of Ladakh as a leafy vegetable and forage [36]. This plant has multiple uses in cuisine as salad, pickle and condiments. *Capparis* is known to contain a wide variety of antioxidant compounds including phenolic compounds. These compounds are found to be well correlated with antioxidant potential.

Previous chemical studies on *C. spinosa* have shown the presence of alkaloids, lipids, polyphenols, flavonoids, indole and aliphatic glucosinolates [210]. Ethanolic extract from the fruit of *Capparis spinosa* exhibits a notable activity in protecting against oxidative stress and suggesting its protective effect against skin sclerosis [163]. However, methanolic extract of *C. spinosa* buds, rich in flavonoids, including several quercetin and kaempferol glycosides, was demonstrated to possess strong antioxidant/free radical scavenging effectiveness, antiviral and immuno-modulatory effects in different *in vitro* tests [153]; whereas based on *in vivo* test this extract showed a noteworthy anti-allergic effectiveness against bronchospasm in guinea pigs [152], and when topically applied it

afforded significant *in vivo* protection against UV-B light induced skin erythema in humans [23]. Natural antioxidants present in *Capparis spinosa* can scavenge harmful free radicals from our body. It is possible to reduce the risk of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants [211].

Until now, there has been no report determining the polyphenol contents and antioxidant activity of edible parts of *Capparis* from Ladakh region. In the present study we investigated and compared the above mentioned parameters of four different edible parts: leaves, buds, fruits and roots. In addition, correlations between all the analysed parameters were evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Plant samples, estimation of total phenolic content and antioxidant activity

The edible parts such as leaves, flower buds, roots and dried fruits were collected in triplicate from *Capparis* plants growing in wild from Ladakh during the month of July 2009. The total phenols and flavonoids are extracted from the plant samples as described previously (chapter 4). Similarly the antioxidant activities of plant samples were studied based on DPPH, ABTS and FRAP assays as described previously (Chapter 4).

5.3 RESULT AND DISCUSSION

5.3.1 DPPH radical scavenging activity

DPPH is one of the compounds that possess a proton free radical and shows absorption band at 517nm in visible region. When DPPH encounters proton radical scavengers, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. Figure 5.1 shows the dose-response curve of DPPH radical scavenging activity of the methanol extracts from all the edible parts of the *C. spinosa*. Methanolic extract of leaves showed the highest free radical scavenging activity (70.76%) at a concentration of 0.1 mg/ml, followed by methanolic extract of flower buds, then roots and least activity was detected from dried fruits, though the

DPPH radical scavenging abilities of the extracts were less than those of BHT (72.09%). The scavenging activity of methanolic extract was in the order of leaves > buds (6-7 mm) > buds (9-12 mm) > roots > fruits. The variation in antioxidant activity of plant extract from edible parts was statistically significant ($p < 0.05$).

IC_{50} value was determined from the plotted graph of scavenging ability against the concentration of methanolic extract of *Capparis*, higher the IC_{50} value signifies less antioxidant activity and *vice-versa*. Table 1 revealed that highest IC_{50} value for DPPH found in dried fruits (0.097 mg/ml) and lowest were found in leaves sample (0.050 mg/ml). The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [236]. Though the DPPH radical scavenging abilities of the extracts were less than BHT, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. This study suggests that the all edible parts of *Capparis* plant possess antioxidant activity.

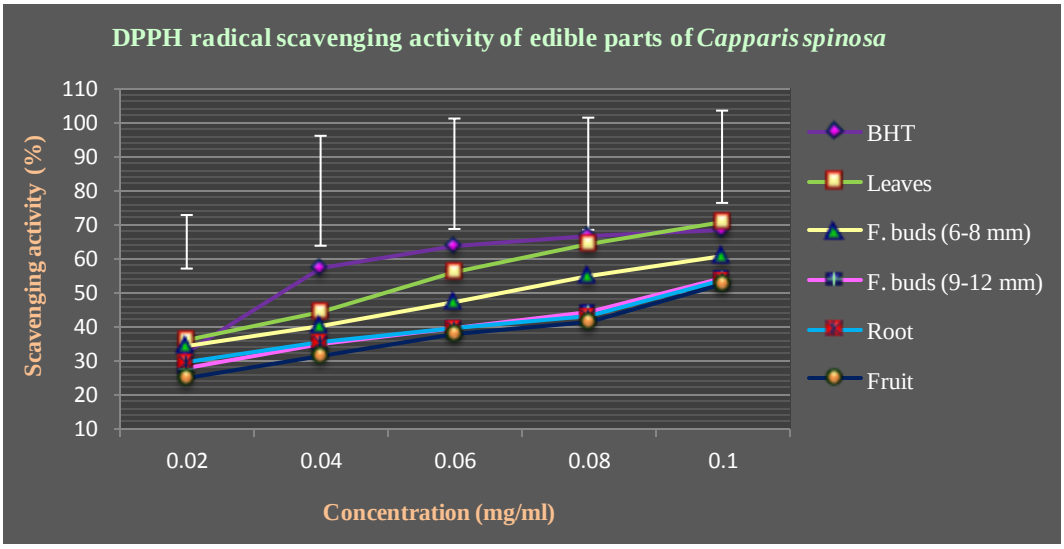


Figure 5.1 DPPH radical scavenging activity of methanolic extracts of all edible parts of *Capparis spinosa* collected from Ladakh region.

Table 5.1 Free radical scavenging activity (IC_{50}) value for methanolic extract of all edible parts of *C. spinosa* collected from Ladakh region.

Edible parts	IC_{50} mg/ml	
	DPPH	**ABTS**
Leaves	0.050 ± 0.003^{a}	0.033 ± 0.003^{a}
F. Buds (6-7 mm)	0.067 ± 0.002^{b}	0.047 ± 0.002^{b}
F. Buds (9-12 mm)	0.091 ± 0.002^{c}	0.077 ± 0.002^{d}
Roots	0.094 ± 0.003^{cd}	0.066 ± 0.003^{c}
Fruits (Dried)	0.097 ± 0.002^{d}	0.076 ± 0.002^{e}

Values are expressed as mean ± standard deviation (n = 3). BHT used as a standard. IC_{50} means with different superscript were significantly different ($p < 0.05$, ANOVA).

5.3.2 ABTS radical scavenging activity

ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals [237]. The methanol extracts of the leaves of *C. spinosa* were fast and effective scavengers of the ABTS radical (Fig. 2) and this activity was comparable to that of Ascorbic acid and BHT. At 0.02, 0.04, 0.06 and 0.07 mg/ml, the ascorbic acid and BHT exhibited higher activity than the leaves extracts, but at 0.1 mg/ml the activity of the leaves extracts was similar to that of ascorbic acid and BHT (100%). Lowest activity was found in dried fruit extract (61.15%) at a concentration of 0.1 mg/ml, whereas the flower buds and roots showed reasonably better antioxidant activity.

The scavenging of the $ABTS^{+}$ radical by the extracts was found to be higher than that of DPPH radical. Factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals [220]. Wang et al. [221] found that some compounds which have $ABTS^{+}$ scavenging activity did not show DPPH scavenging activity, but this is not the case in this study.

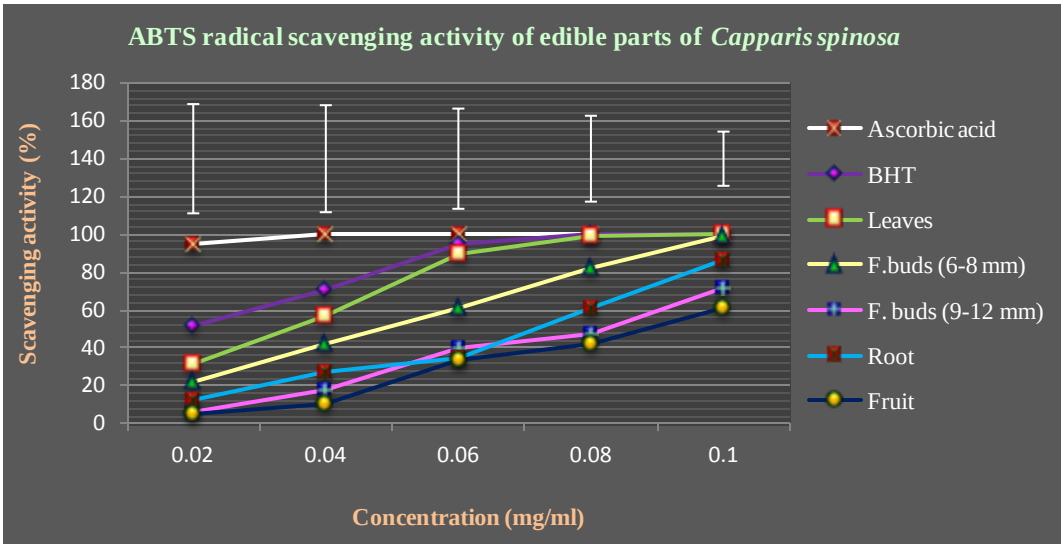


Figure 5.2 ABTS radical scavenging activity of methanolic extracts of all edible parts of *C. spinosa* collected from Ladakh region

5.3.3 Ferric reducing antioxidant power (FRAP) assay

Figure 3 showed that methanolic extract of *Capparis* leaves had highest total antioxidant content (73.54-77.14%) compared to BHT (67.51-76.97%). The lowest percent of antioxidant content was found in dried fruit extract (64.70-71.71) based on the FRAP assay. However, other edible parts are not significantly different in their antioxidant contents.

Generally, the reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain through donating a hydrogen atom [238, 239]. In this study, phenolic compounds of all edible parts of *Capparis* exhibited high reducing power on Fe3+–TPTZ. Rice-Evans [240] reported that phenolic compounds have redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. The redox potential of phenolic compounds plays an important role in determining the antioxidant capacity [240].

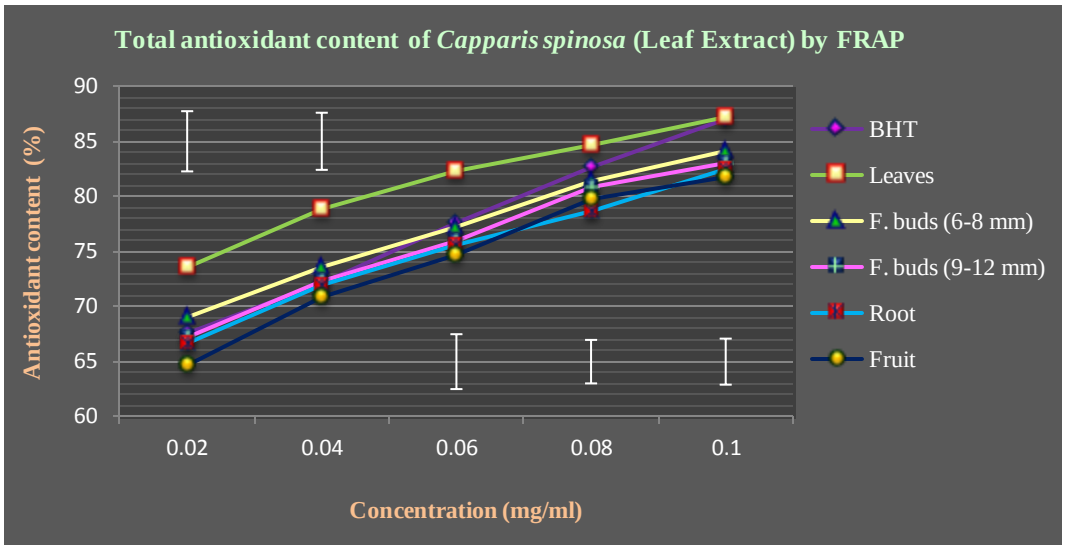


Figure 5.3 Antioxidant content (%) of methanolic extract of all edible parts of *C. spinosa* expressed as percent of antioxidant using FRAP method.

5.3.4 Total phenolic

Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [241, 224]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [225]. The content of phenolic compounds determined by the Folin-Ciocalteu method for the *C. spinosa* leaves analysed is shown in Figure 5.4. Total phenolic compounds ranged from 4.07 to 24.77 mg GAE/g dry wt. The highest total phenolic content was found in the leaves (24.77) followed by flower buds, roots and dried fruits in decreasing order. The lowest phenolic content detected in the dried fruits is 4.7 mg GAE/g dry wt.

In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases [242, 243]. The results strongly suggest that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

5.3.5 Total flavonoids

Figure 5.4 exhibits the flavonoid contents of all edible parts of *C. spinosa***.** The total flavonoid contents in these samples ranged from 0.00 to 5.69 mg quercetin/g DW. The result reveals that leaves having maximum flavonoids contents (5.69 mg quercetin/g dry wt.) and dried fruits having no flavonoid contents (0.00 mg quercetin/g dry wt.) among all edible parts. This result strongly suggests that polyphenol are important components of *Capparis*, which are responsible for not only its antioxidant activities but some of its pharmacological effects could be attributed to the presence of these valuable constituents.

Based on the antioxidant assays, it is thus suggested that phenolic compounds present in the leaves of *Capparis* have strong scavenging ability and ferric reducing power. This could be due to the antioxidant activity of phenolic compounds towards free radicals. Besides, phenolic compounds, the presence of flavonoids might also influence the antioxidant capacity. However, there are several methodological limitations for antioxidant determinations [244].

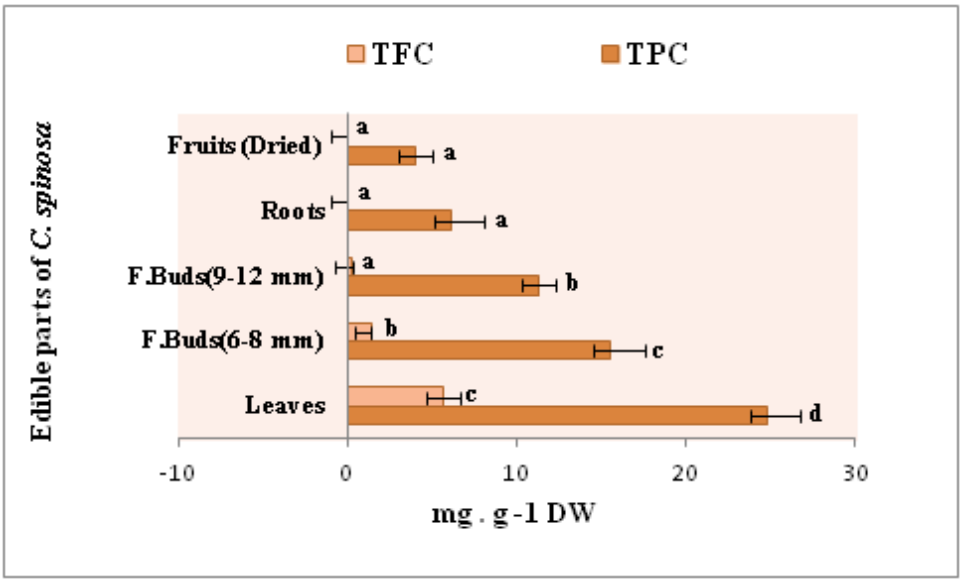


Figure 5.4 Total phenolic content (gray bars, mg of GAE g $^{-1}$ of DW) and total flavonoid content (white bars, mg of quercetin g $^{-1}$ of DW) of *C. spinosa* tender leaves.

5.3.6 Comparative study between total phenolic content and antioxidant activity

Several studies have reported correlations among the antioxidant activities measured by different methods, as well as the correlations between those methods and phytochemical concentrations in various food commodities [218]. However, this type of information is very limited for *C. spinosa*.

Fig. 5.5 shows the comparison among three different antioxidant assays- DPPH, ABTS and FRAP assay. The IC_{50} value of DPPH were highly correlated with IC_{50} value of ABTS (R^2=0.908) (Figure 5.6a) and FRAP assay (R^2=0.977) (Figure 5.6b). The result suggested that the three methods have similar predictive capacity for antioxidant activities of *C. spinosa*. However, IC_{50} value of ABTS reasonably correlated with FRAP assay (R^2=0.5737) (Figure 5.6c). Leong and Shui [252] reported a high correlation (R^2=0.90) between ABTS and DPPH values for various fruit extracts and similarly Lachman et al. [231] reported high correlation (R^2=0.937).

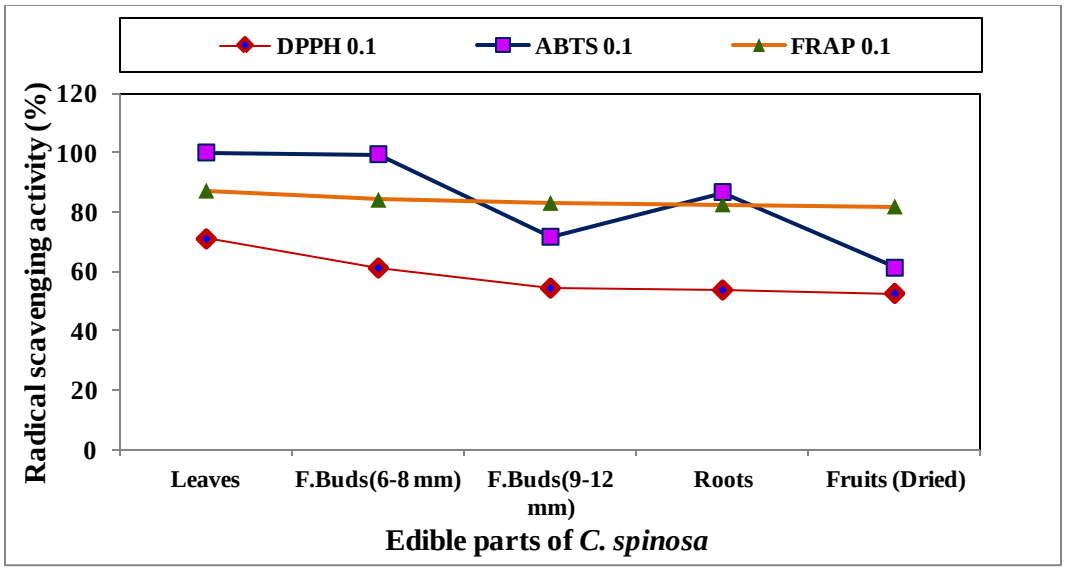
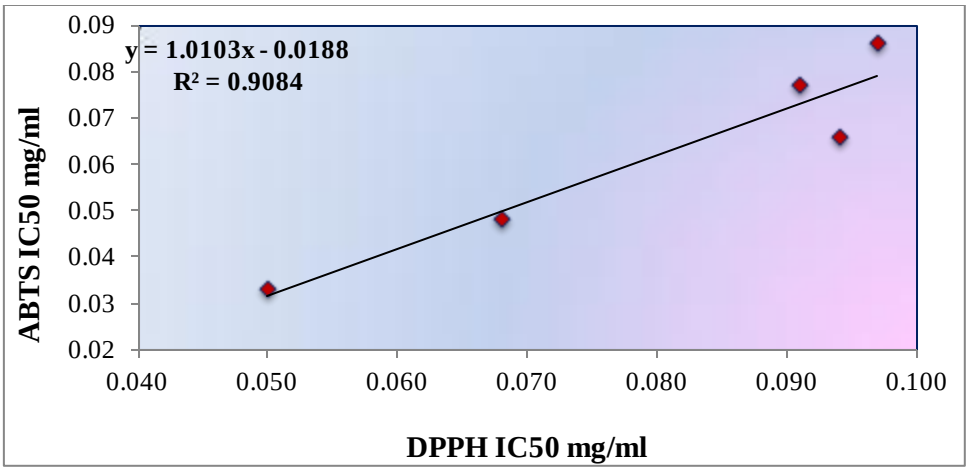


Figure 5.5 Free radicals scavenging activity determined with ABTS, DPPH and FRAP assays at 0.04 mg/ml concentration of methanolic extract of edible parts of *C. spinosa* samples



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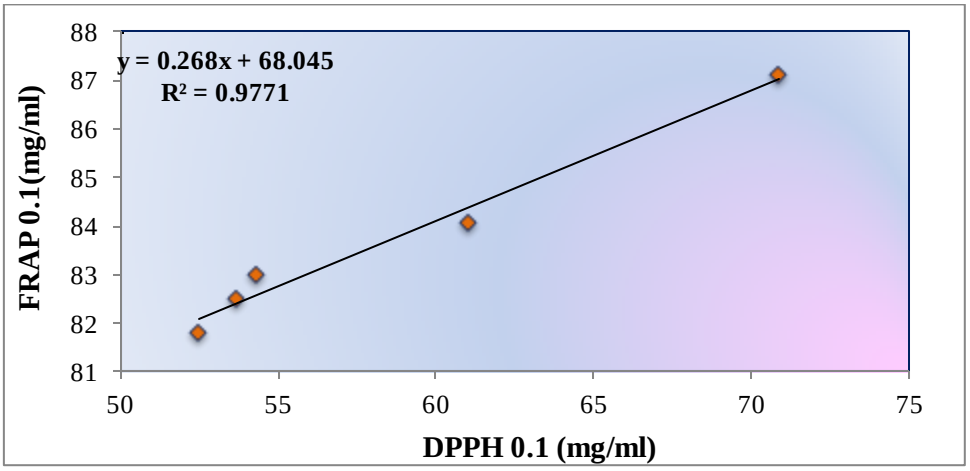

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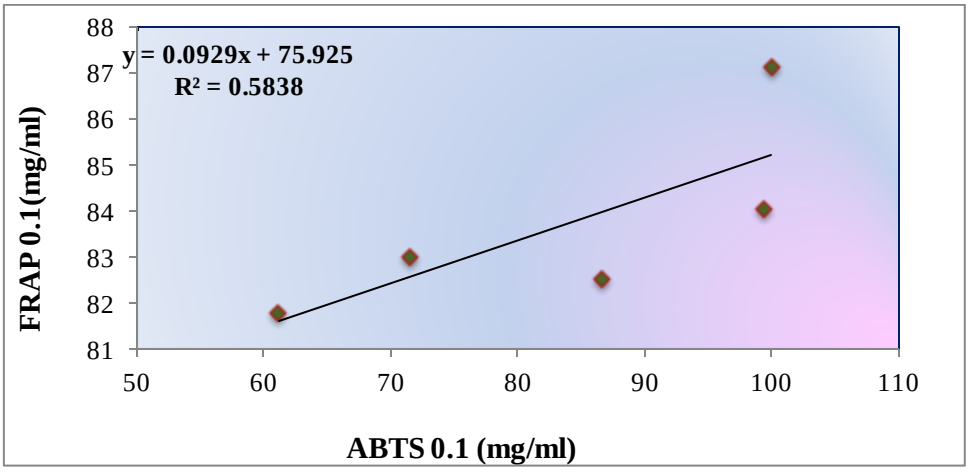

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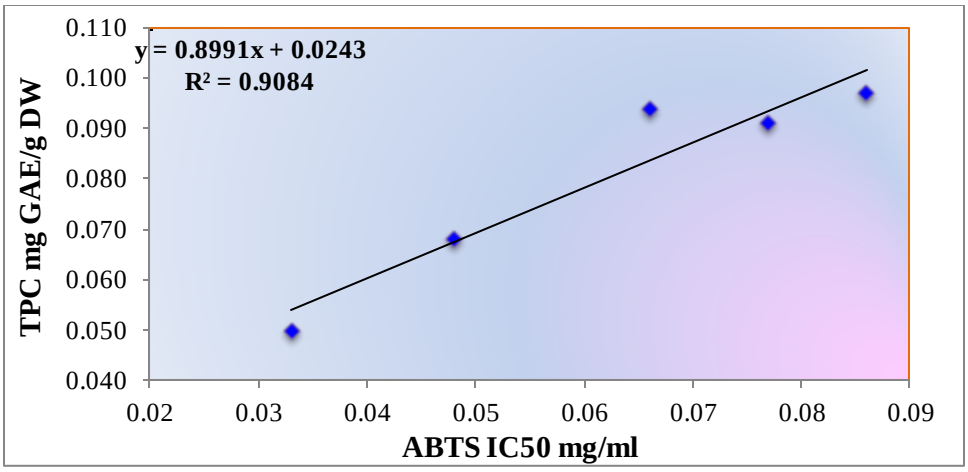
Figure 5.6 Linear correlation between **(a)** DPPH IC_{50} and ABTS IC_{50} **(b)** DPPH 0.1 and FRAP 0.1 **(c)** ABTS 0.1 and FRAP 0.1



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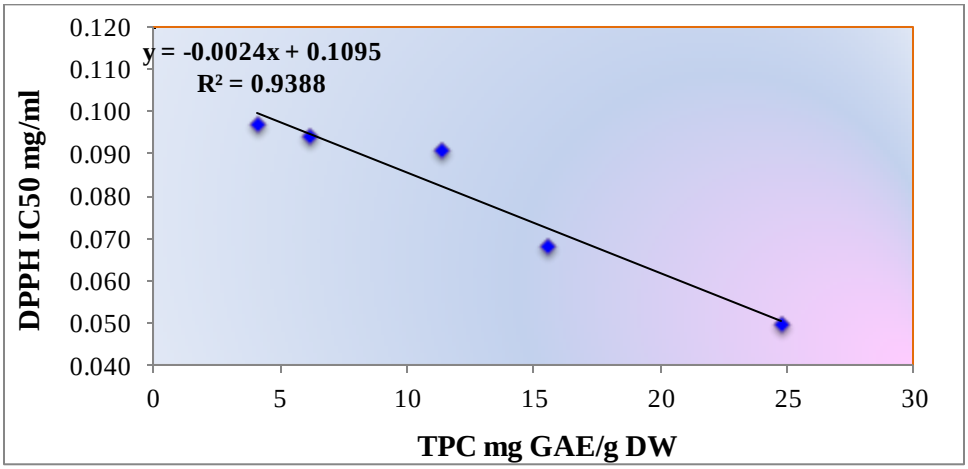

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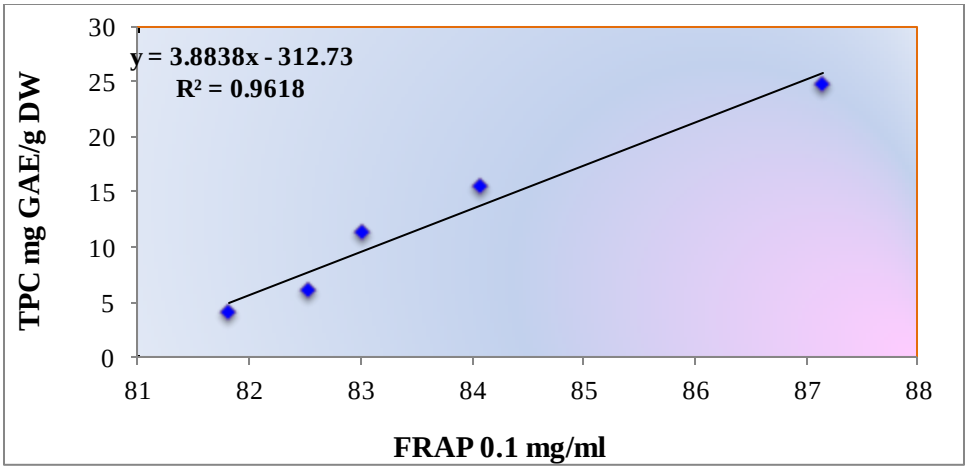

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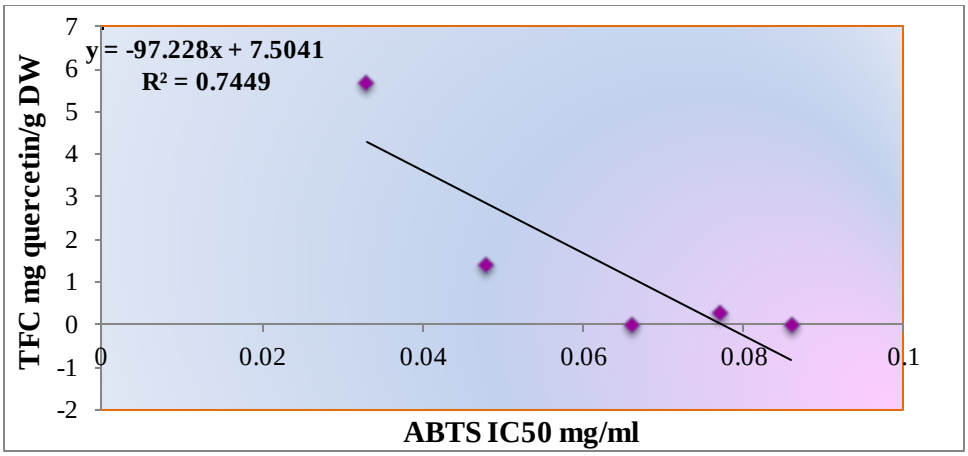
Figure 5.7 Linear correlation between **(a)** TPC and ABTS IC_{50} **(b)** DPPH IC_{50} and TPC **(c)** TPC and FRAP 0.1



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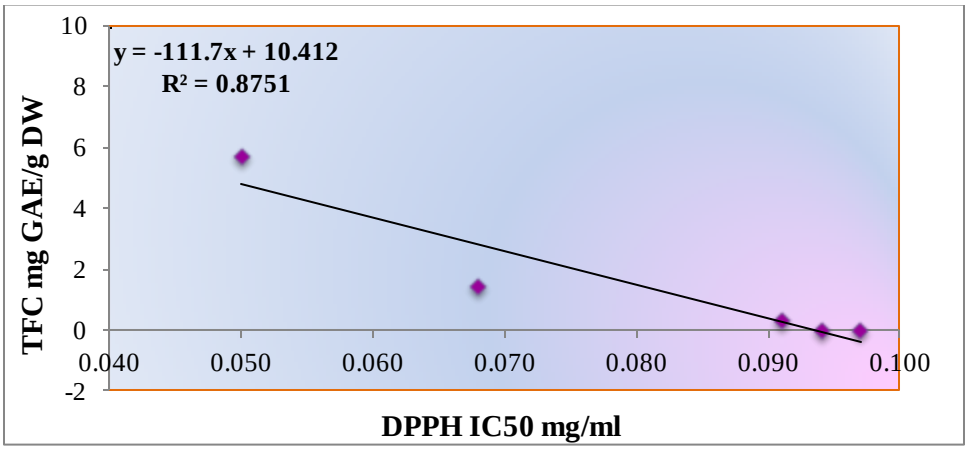

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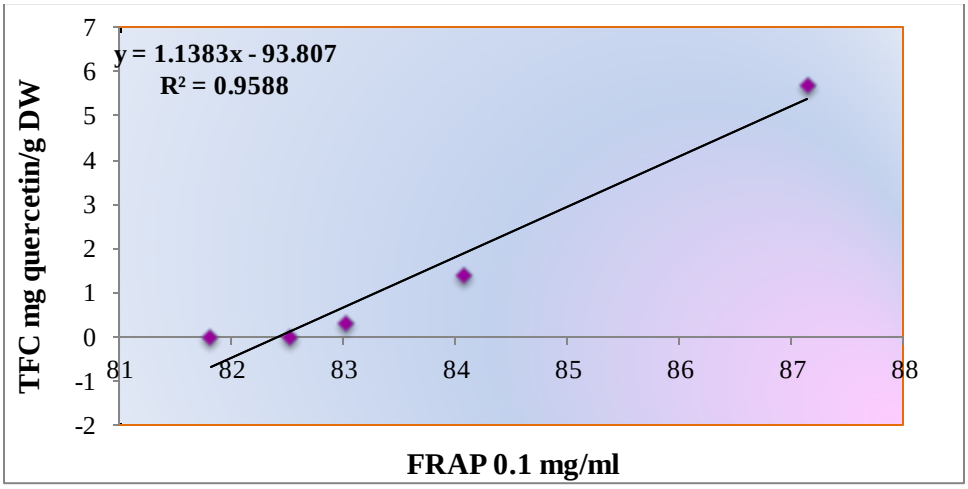


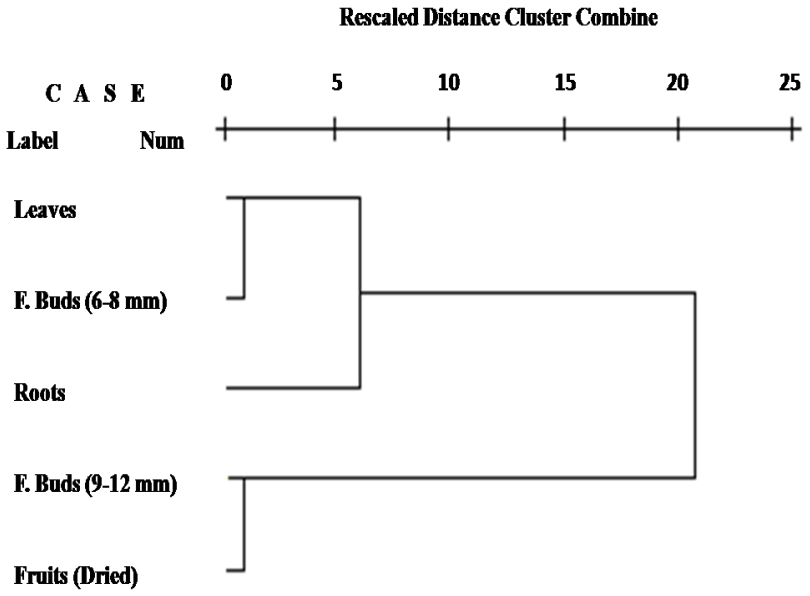
(c)

Figure 5.8 Linear correlation between **(a)** TFC and ABTS IC_{50} **(b)** TFC and DPPH IC_{50} **(c)** TFC and FRAP 0.1

Several studies showed a correlation between antioxidant activity and total phenol contents [226].The total phenolic contents were highly correlated with IC_{50} value of ABTS (R^2=0.908), DPPH (R^2=0.938) and FRAP value (R^2=0.961). This result was in agreement with Benzie and Stezo [245], Othman et al. [232] who found a strong correlation between total phenolics and FRAP assay and Sun and Ho [255] who reported a significant correlation between total phenolics and scavenging ability of buckwheat extract on DPPH radicals. However, total flavonoid contents were reasonably correlated with ABTS (R2=0.745), DPPH (R^2=0.779) but highly correlated with FRAP value (R^2=0.958) of measured value between ABTS and FRAP assay for *Capparis* samples.

5.3.7 Cluster Analysis

Cluster analysis of *C. spinosa* edible parts collected from Ladakh region showed that cluster based on total antioxidant activity (DPPH, ABTS and FRAP) was almost similar to cluster based on total phenolic contents. Where Basgo, Nimmu, Phyang, Batalik samples had fallen in one cluster while samples from Thiksey, Skampuk, Phey, Skuru and Tirchey had fallen in another cluster (Fig. 5.9a and 5.9b). Whereas, in case of cluster based on total flavonoid content samples from Phey and Thiksey are falling in different cluster (Fig. 5.9c). It might be that in case of capers, total AOA activity is more contributed by total phenolics than total flavonoids.



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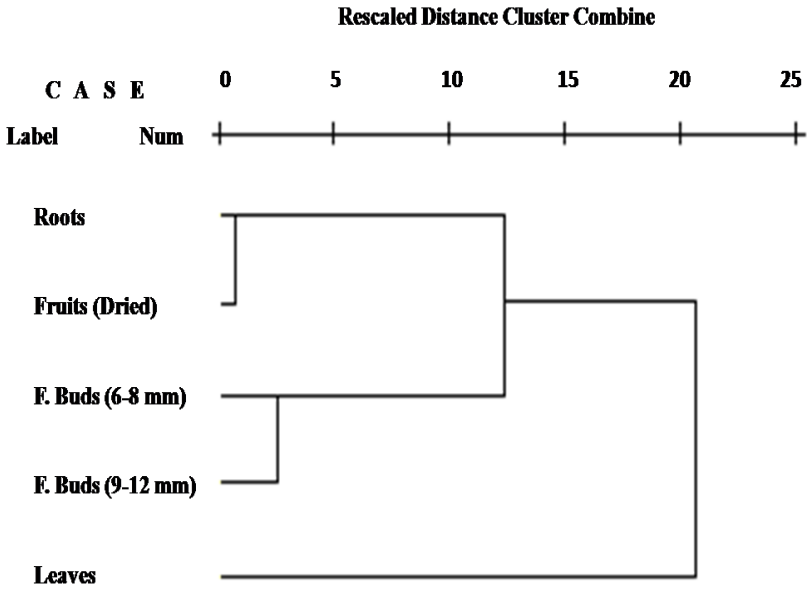

(b)

Figure 5.9 Dendrogram of different sampling sites according to cluster analysis of similarity on the basis of **(a)** Total AOA determined by DPPH, ABTS and FRAP assays **(b)** Total Phenolic and Flavonoid contents using Ward method.

5.4 CONCLUSIONS

Antioxidant activities varied widely among all the edible parts of *C. spinosa*. The highest values of AOA showed unambiguously in the leaves and conversely the lowest values demonstrated in dried fruits. FRAP method revealed that leaves samples possess good antioxidant content comparable with that of the other edible parts. The highest total phenolic and total flavonoid content exhibits in the leaves and lowest in the dried fruits which is same as observed for AOA activity.

There were good correlations among the antioxidant activities measured by DPPH, ABTS and FRAP as well as total phenolic contents, suggesting that these methods have similar predictive capacity for antioxidant activities of edible parts of *C. spinosa*. High correlation between the DPPH, ABTS, FRAP and Phenol and flavonoid contents indicated that the total phenolic contents can be used as indicator for methanolic antioxidant activities of edible parts of *C. spinosa*. This result again suggests that AOA of capers is caused mainly by phenolics and flavonoids.

Thus, the results revealed that the methanolic extracts of *C. spinosa* leaves possess a strong antioxidant/free radical scavenging effectiveness among all the edible parts, which is probably due to the presence of high amount of polyphenolic compounds. The strong antioxidant activity of all edible parts of *C. spinosa* may therefore be a good candidate for functional foods as well as plant-based pharmaceutical products.

CHAPTER 6

ASSESSMENT OF NUTRITIONAL AND PHYSICOCHEMICAL COMPOSITION OF *CAPPARIS SPINOSA* FLOWER BUDS COLLECTED FROM TRANS-HIMALAYA

ABSTRACT

Capparis spinosa (Capparidaceae) is an important under-exploited wild medicinal plant used as leafy vegetable in Ladakh region. Nutritional compositions including carbohydrates, protein, ash, moisture content, minerals and vitamins was compared with reported value. Caper buds collected from the Ladakh region were found to be a good source of protein and minerals as contrast to the reported compositions. The result showed that *Capparis* flower buds are rich in protein (consists of 5.53%), total dietary fibers (3.2%), carbohydrates 12.19%, and total fat content (0.37%). The flower buds are very rich in total calorie value (74.21 kcal per 100 g). It was also found to be very good sources of various minerals. The content of total ascorbic acid, Thiamin, vitamin B-6 obtained was 5.52, 0.05 and 0.12 mg/100g, respectively. Thus caper flower buds could be used effectively as food supplements and also can meet the calorie requirement of local people and the army deployed in the 'Ladakh' sector.

6.1 INTRODUCTION

In the worldwide, more than 7,000 species are cultivated and harvested from the wild. Out of these, there are many food crops that are underutilized or are used only in small communities or areas where the crops are found. The irony is that these unexploited food crops are needed to solve acute food shortages and contribute significantly to agro-biodiversity, food security, nutrition and household income [247]. In India, around 63% of the children fewer than five years of age are malnourished. Nutritionally inferior diets and improper feeding practices are major contributing factors to the development of childhood malnutrition [248]. Various reports noted that many underutilized wild edible plants are nutritionally rich and can supplement nutritional requirements, especially vitamins and micronutrients [249, 250].

The acute imbalance between the increase in the rate of population growth and the world food production has led to the present shortage of nutrients supply and the spread of malnutrition. Available possibilities of bridging the gap between present and future food production level and consumption are the exploitation of new nonconventional food resources and the enrichment of the poor quality foodstuffs. There is a strong linkage between the cultural heritage and the places of origin of the plant species. Local and traditional information on their distribution, biology, agronomy and uses are poorly documented. Some of these underutilized plant species are being threatened in due course of time due to several reasons including lack of knowledge on the part of science, technology, research and development (R&D) and more importantly, their displacement by improved plants, mining activities, bush fires, infrastructure development and overgrazing [251]. These foods could be cultivated on a broader scale and harvested to make the food stock.

The issue of underutilized plant species (UPS) has become a subject for discussion in international, national and academic circles, especially in research area. In definition, UPS is a species that are of minor importance in terms of production, consumption and utilization, and not fully important to contribute to the national

economy. As we look further, these species are not only functioning as food stock but they could have potential benefit to human in agriculture, medicine and industry. In different point of view, UPS have the potential to reduce poverty and alleviate hunger.

The caper bush (*Capparis spinosa*) belongs to family Capparidaceae, commonly known as 'Caper' and locally known as '*Kabra'* is one of such wild vegetable, that grows as an underutilized wild edible plant in all over the Ladakh region, but is a semi-cultivated popular condiment vegetable in many parts of European and other countries. Unopened flower buds are laxative. They are used internally in the treatment of coughs, & externally to treat eye infections. The buds are rich source of aldose-reductase inhibitors and thus are effective in preventing cataracts. The buds are harvested before the flower open and used for making pickle, when prepared correctly they are said to ease stomach pain Also the flower buds and roots are used as renal disinfectants, diuretics, tonic, for arteriosclerosis and as compresses for the eyes [252, 253, 254].

There is very little information published on the physicochemical composition of caper buds all over the world. Till date no report has been published on the nutritional profiling of Caper buds from the cold arid desert of Ladakh. Therefore the aim of this study was to characterize and estimate nutritional composition of *C. spinosa* buds from the Ladakh region for its utilization as food supplements. This data provides a scientific basis for future quality improvement in breeding program.

6.2 MATERIAL AND METHOD

6.2.1 Plant collection

Fresh flower buds of *Capparis* were collected from the plants grown in the Ladakh region in the month of July 2009 for the investigation of nutritional compositions. The plant was identified by its vernacular name by the local people and later authenticated at the Herbarium of Forest Research Institute and Botanical Survey of India, Dehradun, India.

6.2.2 Proximate analysis

Moisture content was determined by digital moisture analyzer (Co. name). Crude fiber was determined by AOAC (255). The estimation of total protein was made by Kjeldahl method and the total fat content of sample was determined by the standard AOAC method (256). The content of total carbohydrate was determined by the following equation:

Carbohydrate (%) = 100- {Moisture (%) + Protein (%) + Fat (%) + Ash (%)}

Ash was determined by heating sample at $600^{O}C$ for six hours or until a constant weight was reached. The gross food energy was estimated (257, 258), using the equation:

FE = (% CHO x 4) + (% CF x 9) + (% CP x 4)

Where FE = Food Energy in Kcal /g, CF = Crude Fat, CP = Crude Protein.

6.2.3 Mineral analysis

6.2.3.1 Sample Preparation for Mineral Analysis:

Flower buds were washed in 0.2 % detergent solution followed by rinsed in deionized water. After decontamination, samples were dried immediately. Samples placed in oven for overnight at 80°C for complete drying of tissue. After complete drying sample were ground by using grinder and sieved it by 20-mesh sieve. After proper sieving and homogenization sample were kept in tight lead container at 4° C.

6.2.3.2 Digestion of sample:

1 g fine powder of flower buds was taken in to 30 mL glazed porcelain crucible. Placed the crucible into a cool muffle furnace and muffle at 500°C for 2 hr. Removed the crucible from the furnace, cool it and add 3.0 mL HNO_3. Heat the sample on a hot plate at 100°C until dry. Place the crucible again into the muffle furnace, let cool it and add 10 mL HCL. Transfer sample to a 50-ml volumetric flask, dilute to volume with deionized water and mix well.

All the element analyzed by using the Inductively Coupled Atomic Adsorption Spectrometer (Perkin-Elmer Optical Emission Spectrometer, Optima 7000 DV),

according to the methods in Handbook of Reference Methods for Plant Analysis by Yash P. Kalra (259) and instruction given by supplier of ICP OES.

6.2.4 Vitamin analysis

The caper buds samples were analyzed for different vitamins composition using High Performance Liquid Chromatography (HPLC). Vitamins from the plant samples were extracted as per the method of Perales *et al.* [260].The chromatographic system was equipped with a Shimadzu HPLC and photodiode array detector. Supelcosil LC 17 DB column (250 mm×4.6 mm, 5µm; Sigma, USA) were used for separation of vitamins. Vitamins composition of the plant samples was investigated in triplicate.

6.3 RESULT AND DISCUSSION

The nutritional composition of *Capparis* flower buds may vary from one continent to another, one country to another in the same continent, and also may vary from region to region. This variation may be due to change of climatic condition, nature of soil, photoperiods, rainfall and other environmental factors. Although the nutritional compositions of *Capparis* from worldwide was reported and included in United State Department of Agriculture (USDA) database, no report is available so far regarding the nutritional compositions of flowers buds of *Capparis* from the Ladakh region. We carried out the various nutritional compositions of flowers buds and compared with the existing data as follows.

6.3.1 Proximate compositions

The proximate compositions of the *Capparis* flower buds from the Ladakh region are shown in Table 1. The moisture content of capers flower bud was found quite low (70.22%) in comparison to the reported value (73.75%), it may be advantageous in view of the samples shelf life. The result showed that *Capparis* flower buds are rich in protein. It consists of 5.53% per 100g of sample that is higher than the reported value (2.36%). Thus capers buds could contribute significantly to the recommended human daily protein requirement. The ash content of caper buds was 1.69% which is lower than that of

reported value (7.04%). The value reported for total dietary fibers (3.2%) is quite higher than value obtained (2.6%). However, the amount of carbohydrates obtained was 12.19%, which is three times higher than that of reported value (4.79%). The total fat content was 0.37%. Fats are essential in diets as they increase the palatability of foods by absorbing and retaining their flavors and help in the transport of nutritionally essential fat-soluble vitamins [261].

Table 6.1 Proximate composition of *Capparis spinosa* flower buds.

Proximates	**Units**	**Obtained value (per 100g)**	**Reported value * (per 100g)**
Moisture	g	70.22	73.75
Energy	kcal	74.21	23.21
Protein	g	5.53	2.36
Ash	g	1.69	7.04
Carbohydrate	g	12.19	4.79
Fiber, total dietary	g	2.6	3.2
Total Fat	g	0.37	0.0

*** USDA Nutrient Database for Standard Reference, Release 13 (1999) NBD No. 02054**

6.3.2 Mineral compositions

Capers are very rich in minerals. The results of the mineral compositions of caper buds are shown in Table 2. The calcium content and iron content was 99.17 mg/100g and 2.30 mg/100g, respectively which is quite double than that of the reported value 40.00 mg/100g and 1.67 mg/100g, respectively. Magnesium, potassium, zinc and manganese obtained in capers was 56.32, 4.95, 0.95 and 0.42 mg/100g respectively, which is higher than that of reported value. However, phosphorus, sodium, copper and selenium obtained in capers was 7.57, 2.79, 0.23 and 0.004, respectively which was quite lesser than that of reported values. Overall the results revealed that caper buds are good sources of minerals and could be used as food supplements.

Table 6.2 Mineral elemental composition of *Capparis spinosa* flower buds

Minerals	**Units**	**Obtained value (per 100g)**	**Reported value * (per 100g)**
Calcium, Ca	mg	99.17	40.00
Iron, Fe	mg	2.30	1.67
Magnesium, Mg	mg	56.32	33.00
Phosphorus, P	mg	07.57	10.00
Potassium, K	mg	4.95	40.00
Sodium, Na	mg	2.79	2.00
Zinc, Zn	mg	0.95	0.32
Copper, Cu	mg	0.23	0.37
Manganese, Mn	mg	0.42	0.07
Selenium, Se	mg	0.004	1.2

6.3.3 Vitamin compositions

Caper buds contain variety of vitamins and could serve as food supplements. The details of vitamin analysis are shown in Table III. The content of total ascorbic acid, Thiamin, vitamin B-6 obtained was 5.52, 0.05 and 0.12 mg/100g, respectively which are quite more than that of reported value of 4.3 mg/100g for total ascorbic acid, 0.01mg/100g for thiamin and 0.02 for vitamin B-6. Folic acid content obtained was 0.176 mcg/100g; however this vitamin was not detected in the reported value.

Table 6.3 Essential Vitamin composition of *Capparis spinosa* flower buds

Vitamins	**Units**	**Obtained value (per 100g)**	**Reported value * (per 100g)**
Vitamin C,	mg	5.52	4.3
Thiamin	mg	0.05	0.017
Riboflavin	mg	0.027	0.139
Niacin	mg	0.132	0.652
Vitamin B-6	mg	0.121	0.023
Folic acid	mcg	0.176	0.0
Vitamin B-12	mcg	ND	0.0
Vitamin A, IU	IU	ND	137.0
Vitamin E	mg	ND	0.77

6.4 CONCLUSION

Caper buds are highly nutritious. But when the nutritional composition of caper buds were compare with nutritional composition of caper buds in Ladakh region then it is found that the caper buds grow in Ladakh region having more nutritional properties than that of the other parts of the world. Therefore the caper bush could be used as a food supplements to nourish the local peoples form the Ladkah region or other parts of India.

CONCLUSION

- Seed germination was increased significantly by different types of treatments suggesting effective breaking of physical and physiological dormancy. The described method will ultimately help in the widespread cultivation of this wonder plant at the farmers' field of Ladakh.

- High genetic variation was observed in the genotypes of *Capparis*, which implied the need to conserve sufficiently large populations in natural habitats for conservation of its genetic diversity and avoidance of genetic erosion.

- The leaves extract of *Capparis* plant contain significant amount of polyphenols and antioxidant activity. The variation in polyphenols and antioxidant activity among different populations is not significantly related with genetic variations measured based on RAPD, ISSR and in combination of RAPD and ISSR markers.

- All the edible parts including leaves of *C. spinosa* also contain significant amount of polyphenolic compounds and possess a strong antioxidant/free radical scavenging activity. The leaves and caper buds contain high amount of polyphenols and antioxidant activity.

- Flower buds of *C. spinosa* grow in Ladakh region having more nutritional contents than the other parts of the world. Therefore it can be used as nutritional supplements.

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