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Estimation of antioxidant activity and total phenolics among natural populations of Caper (*Capparis spinosa)* leaves collected from cold arid desert of trans-Himalayas

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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 (R^2=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 0.408, 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.

Keywords: *Capparis spinosa*, antioxidant activity, polyphenol, trans-Himalayas.
Abbreviation: BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2, 2-azinobis-3- ethylbenzothiazoline-6-sulfonic acid; FRAP, ferric reducing/antioxidant power assay; GAE, Gallic acid equivalent; AOA, Anti Oxidant Activity.

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 (Ames et al., 1993). *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 (Mishra et al., 2009). *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 (Sharaf et al., 2000). 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 (Cao et al., 2010). 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 (Arena et al., 2007); *in vivo* this extract showed a noteworthy anti-allergic effectiveness against bronchospasm in guinea pigs (Trombetta et al., 2005), and when topically applied it afforded significant *in vivo* protection against UV-B light induced skin erythema in humans (Bonina et al., 2002). 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 (Stanner et al., 2000). 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 (Tomaino et al., 2005). 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 quality, data on antioxidant capacity and polyphenol content would be additional information to be

Table 1. Collection sites of *Capparis* leaves (along with altitude) from trans Himalayas of Ladakh

Valley	Sampling sites	Altitude (m)
Suru	Batalik	3310
Indus	Basgo	3320
	Nimmu	3242
	Phyang	3347
	Phey	3185
	Thiksey	3435
Nubra	Skampuk	3197
	Skuru	3117
	Tirchey	3159

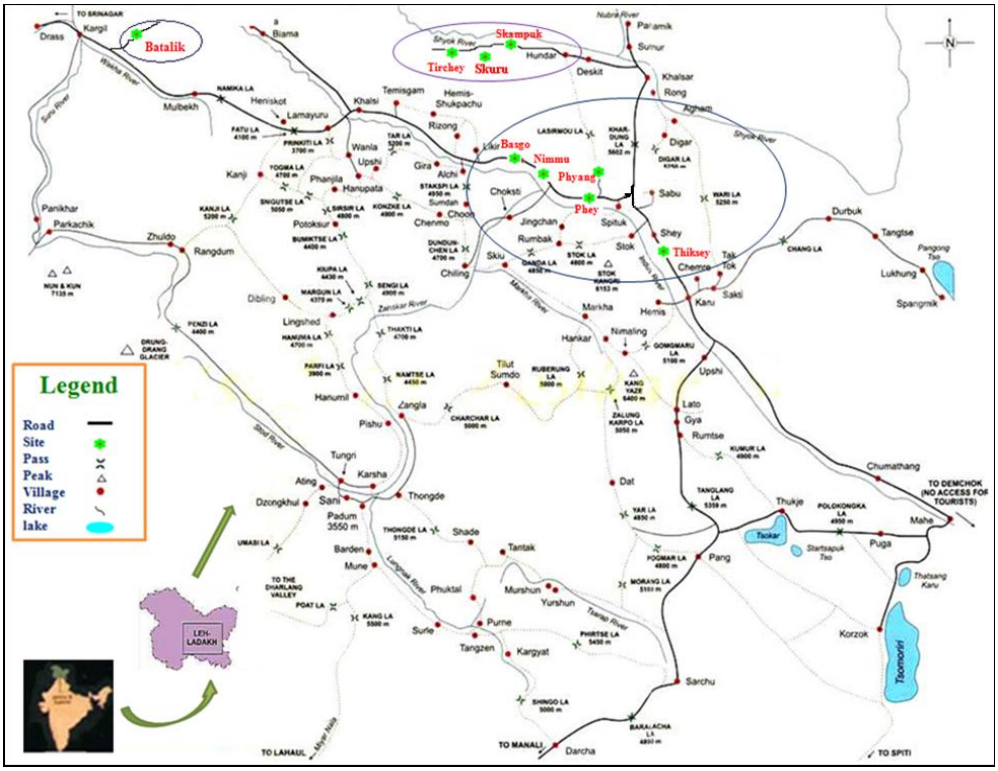


Fig 1. Collection sites of *Capparis* tender leaves from three valleys (Suru, Indus and Nubra) and nine villages located in Ladakh region of Jammu & Kashmir, India

considered when promoting the consumption of caper in different forms.

Result and discussion

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. 2 shows the IC_{50} values of DPPH and ABTS radical scavenging activity of the methanolic extracts of *C. spinosa* leaves, collected from 9 sites of trans-Himalayan region (India). 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* site showed lowest DPPH based IC_{50} (66.0 µg/ml) while *Tirchey* (78.0 µg/ml) had the highest IC_{50} value (Fig. 2). 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 while, samples collected form *Nimmu*, *Phyang* and *Trichey* sites have least antioxidant activity. The average IC_{50} values for DPPH radicals obtained were 73.0 µg/ml. For the ABTS radical, leaves collected from *Skuru* and *Phey* sites showed minimum and maximum IC_{50} values (31.0 µg/ml and 39.0 µg/ml respectively) while values observed for other samples were given in the Fig. 2. This indicated that *Skuru* site have maximum while, samples collected form *Nimmu*, *Skampuk*, *Trichey* and *Phey* sites had least antioxidant activity. The average IC_{50} values for ABTS radicals obtained were 34.0 µg/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. 2). Average IC_{50} values determined by ABTS assay were almost twice lower as compared to the values determined by DPPH assay. Awika et al. (2003) 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

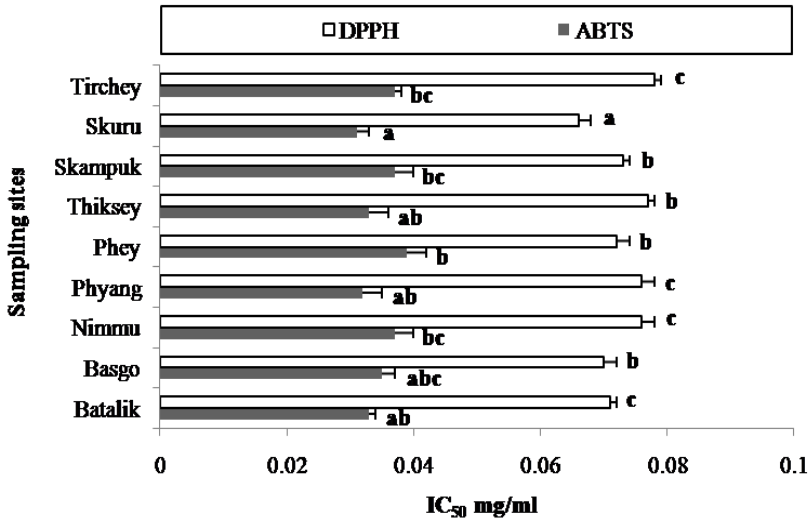


Fig 2. IC_{50} value for DPPH assay (white bars, mg/ml) and ABTS assay (gray bars, mg/ml) of *C. spinosa* leaves collected from different sites in Ladakh region

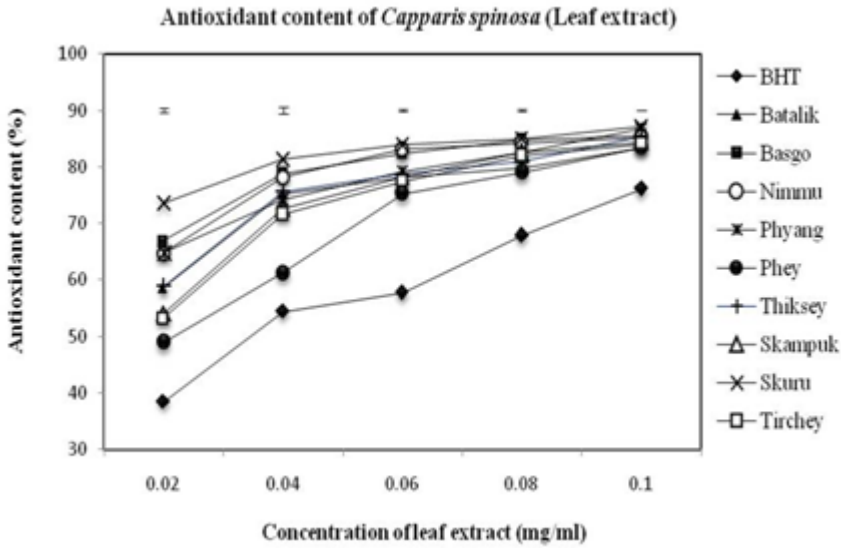


Fig 3. Antioxidant content (%) of *Capparis spinosa* leaves using FRAP assay

values were significantly higher than the DPPH values. Teow et al. (2007) 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 (Yu et al., 2002). Wang et al. (1998) 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.

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 colored complex of ferrous tripyridyltriazine (Fe^{2+}–TPTZ) (Benzie and Strain, 1999). The FRAP assay is widely used in the evaluation of the antioxidant component in the dietary polyphenols (Luximon-Ramma et al., 2005). Highest total antioxidant content (%) was observed in the leaves samples collected from *Skuru* (73.54-87.14%) while, lowest was found in *Phey* (48.97-83.38%) and *Tirchey* (53.26-84.07) samples (Fig. 3). However, not much significant difference was observed for total antioxidant content (%) between other location samples. For BHT (positive control) this value varied between 38.50 to 75.97%. Likewise in DPPH and ABTS assay, FRAP assay also showed lowest antioxidant activity (AOA) for the *Phey* and *Tirchey* while highest for the *Skuru* samples, suggesting that these methods have similar predictive capacity for AOA in *Capparis*. Antioxidant content (%) as determined by FRAP assay ranged from 83.43 to 87.14% at 0.1 mg/ml of leaf extract and increase in AOA was almost similar in DPPH and ABTS assay (Fig. 4).

Total phenolic

Phenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds (Tepe et al., 2006). 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 (Zheng and Wang, 2001). 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*).

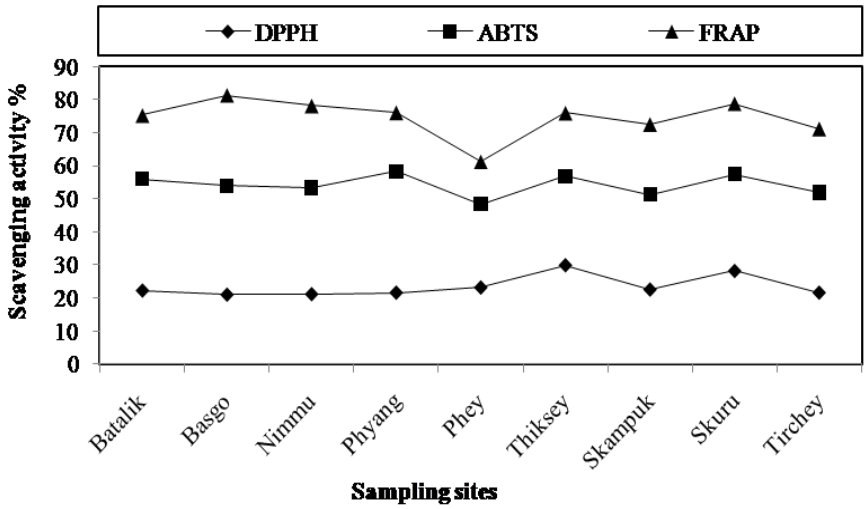


Fig 4. Free radicals scavenging activity determined with ABTS, DPPH and FRAP assays at 0.04 mg/ml concentration of methanolic extract of tender leaves of *C. spinosa*

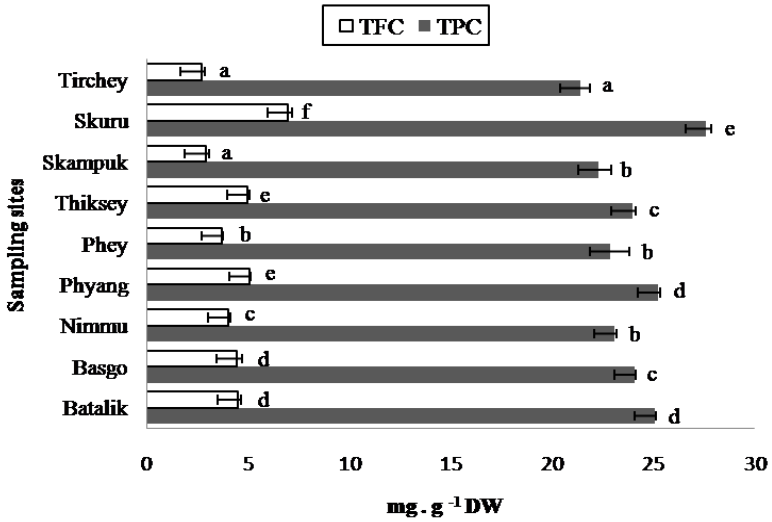


Fig 5. Total phenolic content (gray bars, mg of GAE g $^{-1}$ of DW) and total flavonoid content (white bars, mg of quercetin g $^{-1}$ of DW) of *C. spinosa* tender leaves

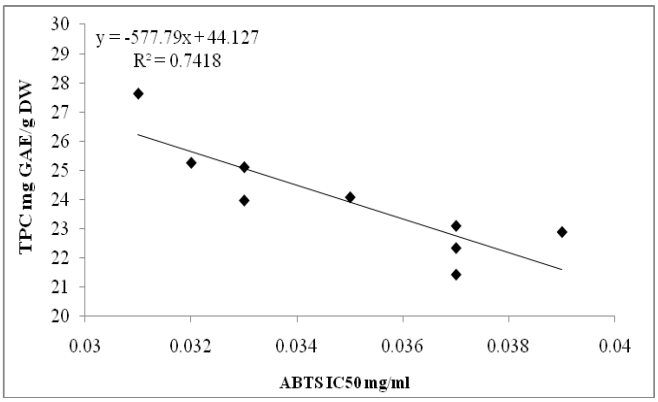
The samples collected from *Nimmu*, *Phey*, *Skampuk* and *Thicksey*, *Basgo* and *Batalik*, *Phyang* sites were not significantly different ($p<0.05$) from each other (Fig. 5). This correlate with the finding that *Skuru* sample have maximum antioxidant activity while samples collected form *Trichey* 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 (Nagai et al., 2003). Genetic factors and growing conditions may play an important role in the formation of secondary metabolites, including phenolic acids (Hashempour, et al., 2010; Islam et al., 2003). According to Oktay et al. (2003), a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species.

Total flavonoids

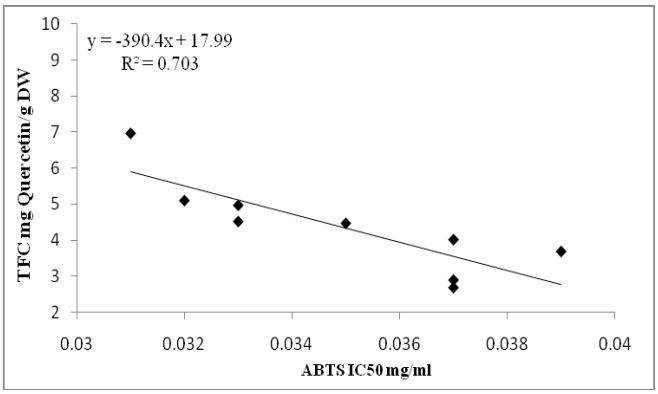
The flavonoid content of *C. spinosa* leaves was shown in Fig. 5. Among the samples analysed, *Skuru* and *Tirchey* samples were found to contain maximum and minimum flavonoid (6.96 and 2.6 mg quercetin equivalent/g DW respectively). Based on the antioxidant assays, it is thus suggested that phenolic 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.

Correlation

From Fig. 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

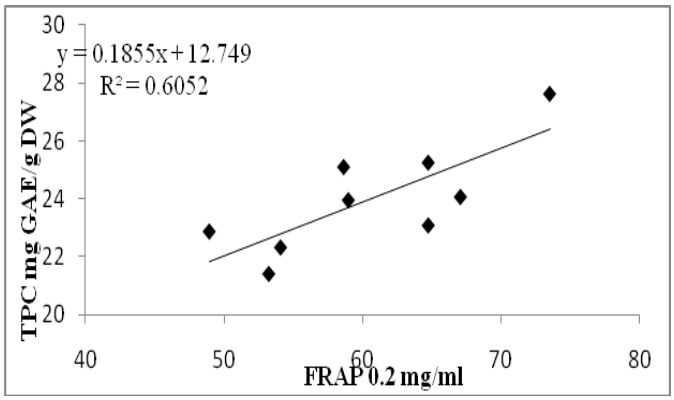


(A)

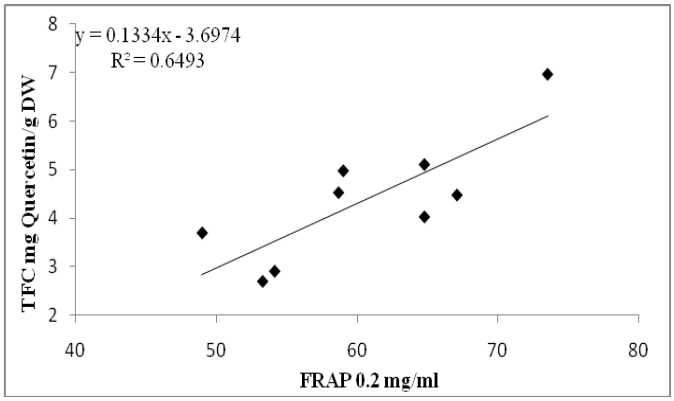


(B)

Fig 6. Linear correlation between (a) ABTS IC_{50} and TPC **(b)** ABTS IC_{50} and TFC



(A)



(B)

Fig 7. Linear correlation between (a) FRAP assay (0.02 mg/ml) and TPC **(b)** FRAP assay (0.02 mg/ml) and TFC

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 (Prior et al., 2005), 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. The results suggested that 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 trans-Himalayan region were relatively lower than those found for other food commodities. Leong and Shui (2001) reported a high correlation (R^2=0.90) between ABTS and DPPH values for various fruit extracts. While, Lachman et al. (2010) observed 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. 6a, 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. 7a, b). This result was in agreement with Othman et al. (2007) 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 (R^2=0.303 and R^2=0.408, respectively). Islam et al. (2003) reported a relatively lower, but significant correlation coefficient (R^2=0.38) 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; Rabah et al., 2004) and cactus pear (R^2=0.970; Stintzing et al., 2005). Therefore, total phenolic content can be used as an indicator in assessing the antioxidant activity of fruits and vegetables. Prior et al. (2005) recommended that the Folin-Ciocalteu method for total phenolic determination can be standardized for comparison of the result between laboratories. 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 for antioxidant activity between genotypes.

Cluster Analysis

Cluster analysis revealed that the cluster based on total antioxidant activity (DPPH, ABTS and FRAP) and total phenolic content are almost similar where *Basgo*, *Nimmu*, *Phyang*, *Batalik* samples had fallen in one cluster while

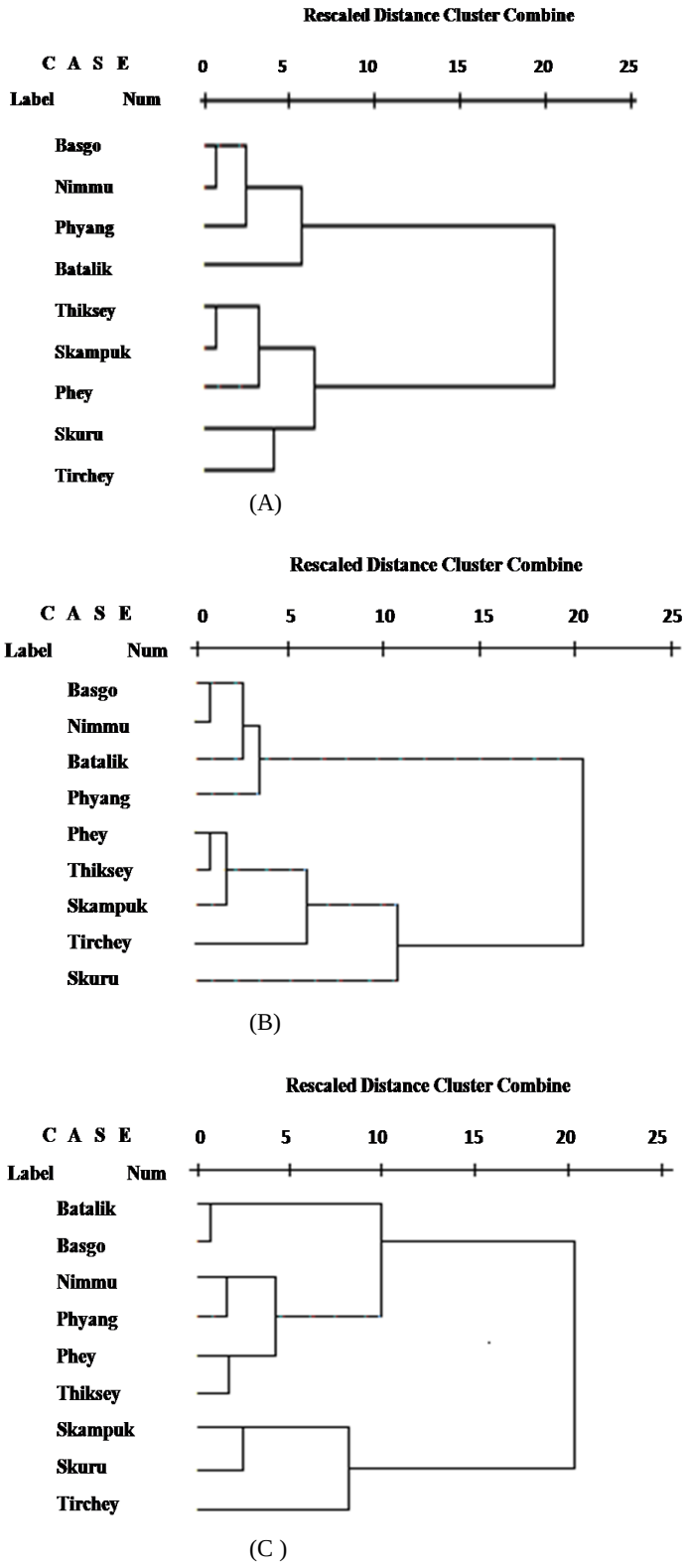


Fig 8. Dendrogram of different sampling sites according to cluster analysis of similarity using Ward method on the basis of (a)Total AOA determined by DPPH, ABTS and FRAP assays (b) Total Phenolic content (c) Total Flavonoid content where, distance is Euclid distance

samples from *Thiksey*, *Skampuk*, *Phey*, *Skuru* and *Tirchey* sites had fallen in another cluster (Fig. 8a and 8b). However, in case of cluster based on total flavonoid content, samples from *Phey* and *Thiksey* are falling in different cluster (Fig. 8c). It suggests that in capers, total AOA is contributed more by total phenolics than total flavonoids.

Materials and methods

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 1, Fig. 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"–78º41'34"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.

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)., $FeCl_3$, ascorbic acid, and butylated hydroxyl toluene (BHT) from HIMEDIA Laboratories Pvt. Ltd. (Germany); Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate (Na_2CO_3), 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.

Determination of antioxidant activity

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 40ºC using a rotary evaporator. Each extract was resuspended in methanol to make 50 mg/ml stock solution.

DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana- Pathiranan and Shahidi (2005). 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.

ABTS radical scavenging assay

ABTS radical scavenging assay was determined according to method of Re et al. (1999). 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%.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted using method of Wong et al. (2006). 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 $_{Sample}$]x100.

Determination of total Phenolics

Total phenol content in the extracts was determined by using modified Folin-Ciocalteau method (Wolfe, 2003). 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).

Determination of total Flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon et al. (2006). 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=0.9812), where x was the absorbance and y was the quercetin equivalent (mg/g).

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-values<0.05 were regarded as significant.

Conclusion

Analysis of AOA by DPPH and ABTS method of methanolic extract of *C. spinosa* leaves collected from different sites in trans-Himalayan region showed differences in antioxidant properties in individual samples of leaves. The highest values of AOA showed unambiguously in the leaves collected from *Skuru* and conversely the lowest values demonstrated in leaves collected from *Tirchey* and *Phey* sites. FRAP method revealed that samples from *Skuru* possess good antioxidant content comparable with that collected from other localities. The highest total phenolic and total flavonoid content exhibits in the leaves collected from *Skuru* while lowest in the *Tirchey* site which is same as observed for AOA activity. The IC_{50} value of ABTS were quite reasonably correlated with FRAP (R^2=0.517) while, DPPH IC_{50} values were poorly correlated with both ABTS (R^2=0.100) and FRAP (R^2=0.223). The total phenolic content and total flavonoid content were found 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) while poorly correlated with DPPH IC_{50} value (R^2=0.303 and 0.408 respectively). This result again suggests that AOA of capers is caused mainly by phenolics and flavonoids. By comparison of three AOA 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, the results from this study indicate that the methanolic extracts of *C. spinosa* leaves possess a strong antioxidant/free radical scavenging effectiveness, which is probably due to the presence of polyphenolic compounds. Thus, *C. spinosa* tender leaves may therefore be a good candidate for functional foods as well as plant-based pharmaceutical products.

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