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SEASONAL VARIATION IN SOME MEDICINAL AND BIOCHEMICAL INGREDIENTS IN *MENTHA LONGIFOLIA* (L.) HUDS.

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Abstract

Shoots of *Mentha longifolia*, collected during different seasons from a natural habitat (Knotti Garden in the Soone Valley of the Salt Range), were evaluated for some key medicinal/biochemical ingredients. Plant samples were analyzed for dry matter, fiber, fat, protein, net free energy (NFE), nitrogen free extractable substances (NFES), macro- (Na, N, Ca, K, P) and micro-nutrients (Zn, Mg, Fe, Cu), and alkaloid, flavonoid and phenolic contents. Total fats, proteins, NFE, NFES, macronutrients (Na, N, Ca, K and P), alkaloids, flavonoids and phenolics generally increased while dry matter, fiber, total minerals, and micro-nutrients (Zn, Mg, Fe and Cu) decreased with increasing maturity of plants in autumn followed by winter. The multivariate analysis (RDA) revealed a significant correlation of most of the biochemicals analyzed such as fat, protein, NFE, phenolics, flavonoids and certain minerals such as N, P and Ca with the winter season. In contrast, dry matter, total mineral, total fiber, and Cu and Fe were strongly influenced by the summer season. However, Mg and Zn contents were similarly affected by both autumn and summer. The autumn season had the least effect on the biochemical ingredients of *Mentha* and only moisture, K and alkaloid contents were associated with this season. The NFES and Na contents showed a slight correlation with each of the seasons as they were almost uniformly influenced by autumn as well as winter. Such temporal variations in biochemical ingredients appeared to be correlated with plant maturity, soil moisture contents and temperature effects during different seasons. It was concluded that the best harvesting season for maximum medicinal ingredients was winter followed by summer and the autumn season was least effective in this regard.

Introduction

Plant diversity has a considerable importance as a source of pharmaceutically active substances (Principe, 1991; Pearce & Puroshothaman, 1992; Samant & Mohinder, 2003; Shrestha & Dhillion, 2003; Aggimarangsee *et al.*, 2005; Thomas *et al.*, 2008). The term ethnobotany was coined by Harshberger in 1896 for the plants used by primitive and aboriginal people for the cure of a number of diseases (Plotkin, 1991). Now the meaning of this term has been considerably broadened and ethnobotany is considered to be a part of economic botany, which emphasizes the economic utilization of plants for human welfare (Heiser, 1993; Wickens, 2001).

Mentha longifolia (L.) Huds. belongs to Lamiaceae (mint family) and is an aromatic perennial herb that grows mostly in semi-shady places on moist soils (Qaiser & Nazimuddin, 1981; Shinwari & Chaudheri, 1992; Ibrar *et al.*, 2007; Sher & Khan, 2007; Shinwari *et al*., 2011). Its leaves or fresh shoots are mostly used as peppermint-scent and for flavoring in salads and cooked foods (Facciola, 1990). *Mentha longifolia*, like other members of this genus, is mostly used as a domestic herbal remedy, especially due to its antiseptic properties and beneficial effects on the digestion (Karousou *et al.*, 2007). The shoots in flowering season possess considerable antiasthmatic, antispasmodic, carminative and stimulant effects (Chopra *et al.*, 1986). A tea prepared from its leaves is used in the treatment of fevers, headaches, digestive disorders and various minor diseases (Foster & Duke, 1990).

Different medicinal plant species show a marked variation in active ingredients during different seasons; these have been widely attributed to variations in environmental variables such as temperature and rainfall (Reddy & Reddy, 1997; Ghimire *et al.*, 2006; Kumar *et al.*, 2007; Ahmad *et al.*, 2008; 2009). In addition, there is little or no knowledge among local harvesters for choosing an appropriate time for harvesting to maximize the medicinal potency of plants such as *M. longifolia* (Shinwari & Gilani, 2003). Hence, the major objective of this study was to evaluate this species for variation in medicinal ingredients of plants growing under natural conditions during different seasons. In addition, this study also aimed to identify suitable harvesting times for the harvest of optimal levels of medicinal components from *M. longifolia*.

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Material and Method

Study area: Knotti garden is located at 32° 40' N and 72° 14' E with an elevation of 783m above sea level. The study area was surveyed during different seasons. *Mentha longifolia*, a clonal species of mint, was found growing during summer, autumn and winter, whereas they were not found during the spring season. Only shoots of this plant species are used for medicinal purposes, so 15 shoots of approximately the same size were collected from four different locations of the study site, and each location was considered a replicate. During different seasons, the sampling was done on the same pattern from different plants of the same clonal population. Thus, the final number of samples was 12. The samples were collected from the study site and brought to the Department of Botany, University of Agriculture Faisalabad for biochemical analysis.

Determination of physicochemical parameters: Shoot moisture contents**,** dry matter, crude fibers, mineral contents, fat contents, nitrogen free extractable substances (NFES) and net free energy (NFE) were calculated using the formulae given by AOAC (Anon., 1984; 1990).

Determination of macro- and micro-nutrients: The multiple stems collected from each location were pooled and dried in an oven at 65°C to a constant dry weight. Oven dried and ground plant material (0.1 g) was digested by the wet digestion (H_2SO_4-H_2O_2) method as described by Wolf (1982). The concentrations of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) were determined using a flame photometer (Jenway PFP-7), whereas those of iron (Fe^{2+}), manganese (Mn^{2+}), zinc (Zn^{2+}) and magnesium (Mg^{2+}) were determined using an atomic absorption spectrophotometer (AAnalyst-300, Perkin Elmer, Germany). Shoot nitrogen was determined using the micro-Kjeldahl method (Bremner, 1965), and phosphorus contents were estimated spectrophotometerically following the methods of Jackson (1962).

Total phenolic contents: Phenolic contents were determined spectrophotometerically as described by Julkunen-Tiitto (1985). Ten ml of 80% methanol were added to one gram plant material (dried and ground). The mixture was shaken vigorously for 2 h on an orbital shaker, then heated at 40-45 °C for five minutes and filtered using Whatman No. 1 filter paper. In order to extract alkali soluble phenolics, the residue left on filter paper was washed with 20 % sodium carbonate and again filtered with Whatman No. 1 filter paper. The methanol phase was then evaporated through a rotary evaporator and the residue was mixed in 10 ml distilled water. The final volume of the solution was made up to 50 ml using distilled water. One ml of 20% sodium carbonate solution was added to 1 ml of the phenolic extract and incubated for 5 min. at room temperature. Then 0.5 ml of Folin Ciocalteo phenol reagent (1:1 diluted with distilled water) was added and incubated for 10 min. at room temperature. Absorbance of the colored solution was read at 735 nm using a spectrophotometer (Hitachi U-2000). The quantity of total phenols was calculated using a standard curve developed with different concentrations of tannic acid (20-50 µg/l) following the above method of color development used for phenolic extract. The results were expressed as mg phenols per g dried weight.

Total flavonoid contents: The flavonoid contents of the shoot samples were determined using the standard method of Dewanto *et al.*, (2002). Dried and ground shoot material (0.1 g) was placed in a 25 ml volumetric flask containing a mixture of 5 ml distilled water and 0.3 ml of 5% $NaNO_2$ solution (Merck). The mixture was vigorously vortexed and allowed to stand for 5 min. Afterwards, 0.6 ml of 10% $AlCl_3.6H_2O$ solution (Merck) was added, and the mixture was vortexed again and allowed to stand for a further 5 min. Then 2 ml of 1 *M* NaOH solution (Merck) were added and the volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance read immediately at 510 nm using a spectrophotometer (Hitachi U-2000). The concentration of flavonoids was calculated using a standard calibration curve using known concentrations of epicatechin (200-800 mg/l). The results were expressed as mg/g equivalent to epicatechin.

Total alkaloid contents: The alkaloidal contents of all samples were determined gravimetrically using the method of Harborne (1973), optimized by comparison with the spectrophotometric method of Sreevidya and Mehrotra (2003). Dried and finely ground material (5g) was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol were added. The beakers were covered with aluminum foil and allowed to stand for 4 h. The entire solution was filtered Whatman No. 1 filter paper and the extract was concentrated in a water bath to one-quarter of the original volume. Then concentrated ammonium hydroxide was added drop by drop to precipitate alkaloids until precipitation was complete and fumes stopped evolving. The whole solution was allowed to settle and the precipitate was collected on oven-dried and previously weighed filter papers. After washing with dilute ammonium hydroxide solution, the precipitate was dried in an oven at 40-45 °C until a constant weight was obtained. The alkaloid precipitates were calculated as mg/g of the dry plant material.

Statistical analyses: Data recorded for all variables were analyzed by Redundancy Analysis (RDA) using Canoco for Windows version 4.5 (2002). The data for all growth, proximate and biochemical parameters recorded in this study were standardized before RDA analysis. The spring season when no *Mentha* plants were found growing was excluded from analysis. Then a multivariate direct gradient model was fitted and all variables were plotted on RDA Axis 1 and 2 using CanoDraw for Windows version 4.0 (2002). For this purpose, the data were normalized by marking all environmental variables (seasons) as nominal. The univariate analysis of variance (ANOVA) was computed using a COSTAT computer package (CoHort Software, 2003, Monterey, California) and used to calculate least significant difference (LSD) values for the comparison of means following Steel *et al*., (1997).

Results

The summary of the results obtained from RDA is shown in Table 1. RDA axis 1 contributed 43.1% towards the total variance while the contribution of axis 2 was 28.2%. Axes 3 and 4 contributed 10.4% and 6.5% towards total variance, respectively. The species-environment correlations along the RDA axes 1 and 2 were 0.968 and 0.988, respectively. The sum of all canonical eigenvalues was found to be 0.713 (Table 1).

The results of this study revealed significant differences for dry matter, total moisture, fibers and minerals in the shoots of *M. longifolia* during different seasons. The variations in these attributes generally appeared to be strongly affected by an increase in plant age as well as changes in environmental conditions during different seasons. The maximum dry weight, total fibers and minerals were recorded during the summer season, while moisture contents were higher than the autumn season. The least dry matter was produced in autumn, total fibers and minerals in winter and moisture contents in summer season (Figs. 1 a, b, c & d).

Table 1. Summary of the RDA showing the effect of seasons on biochemical attributes of *Mentha longifolia* (L.) Huds. shoots collected from Soone Valley of Salt Range.

Parameters and data	Axes				F-ratio	*P* value
	1	2	3	4		
Seasons						
Eigenvalues	0.431	0.282	0.104	0.065	7.450	0.0020
Species-environment correlations	0.968	0.988	0.000	0.000		
Cumulative percentage variance						
Of species data	43.1	71.3	81.7	88.2		
Of species-environment relation	60.4	100.0	0.0	0.0		
Sum of all canonical Eigenvalues	0.713					

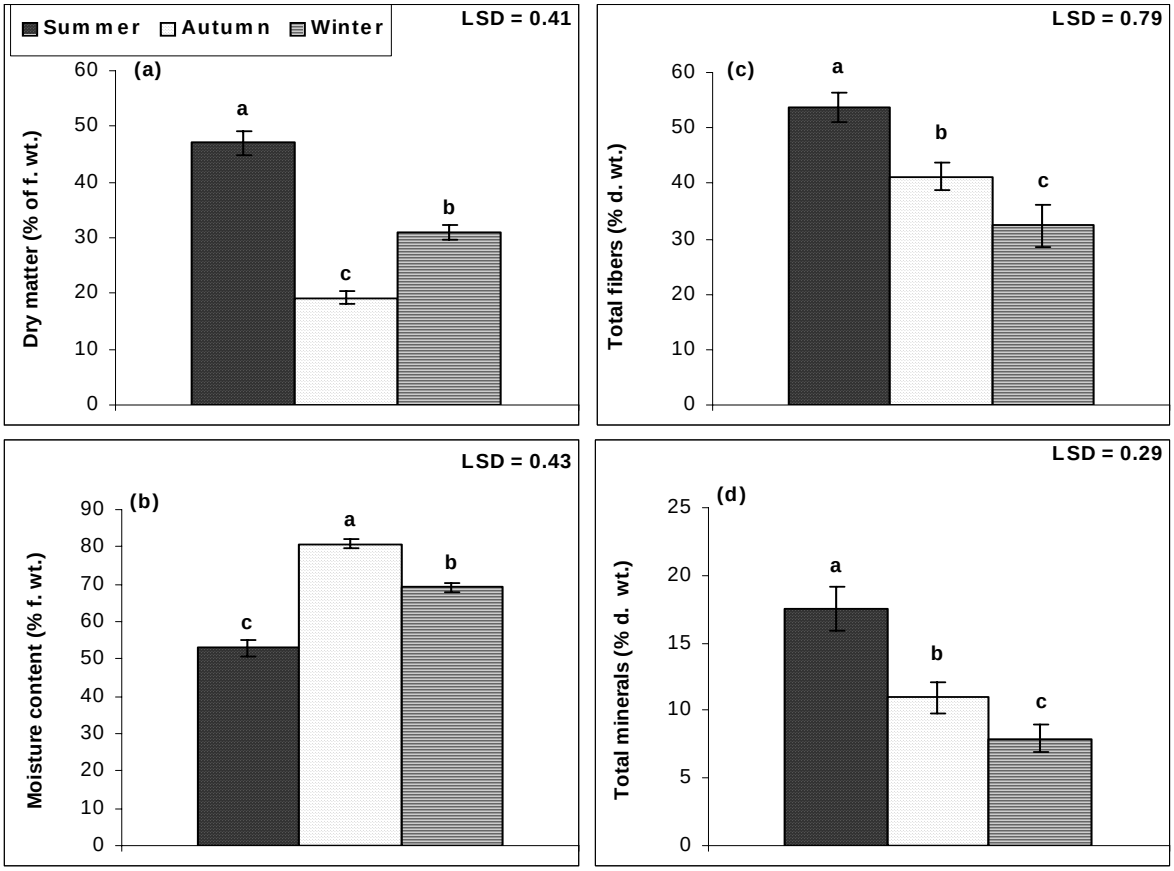

Fig. 1. Seasonal variation in (a) dry matter, (b) moisture content (c) total fibers, and (d) total mineral contents in the shoots of *M. longifolia* at Knotti Garden (f. wt. = fresh weight, d. wt. = dry weight).

The shoots of *M. longifolia* collected during different seasons differed significantly for total fats, proteins, nitrogen free extractable substances (NFES) and net free energy. All these parameters were the highest at the time of maturity of plants during the winter season. The lowest values of NFES and NFE were recorded during the period of sprouting and early growth stages, and they increased considerably with the maturity of plants in autumn and were the highest in winter. However, fat and protein contents decreased in autumn, although these parameters subsequently increased significantly during the winter season at plant maturity (Figs. 2 a, b, c & d).

Micro- and macro-nutrients underwent considerable changes through autumn and winter seasons. The sodium, calcium, nitrogen and phosphorus contents increased as the plants matured and the maximum contents were recorded in plants harvested in winter. However, potassium contents first increased in the autumn but then K decreased significantly during the winter (Figs. 3 a, b, c, d & e). On the other hand, zinc, magnesium, iron and copper contents decreased consistently as the plants aged and the lowest values were observed during the winter (Figs. 4 a, b, c & d).

Total phenolics, alkaloids and flavonoids also varied significantly during different seasons. The phenolic contents in the shoots of *M. longifolia* plants increased consistently with maturity. However, total alkaloids increased significantly during autumn followed by a decrease during the winter season. The reverse was true for flavonoids, which decreased in autumn but increased significantly during the winter season (Figs. 5 a, b & c).

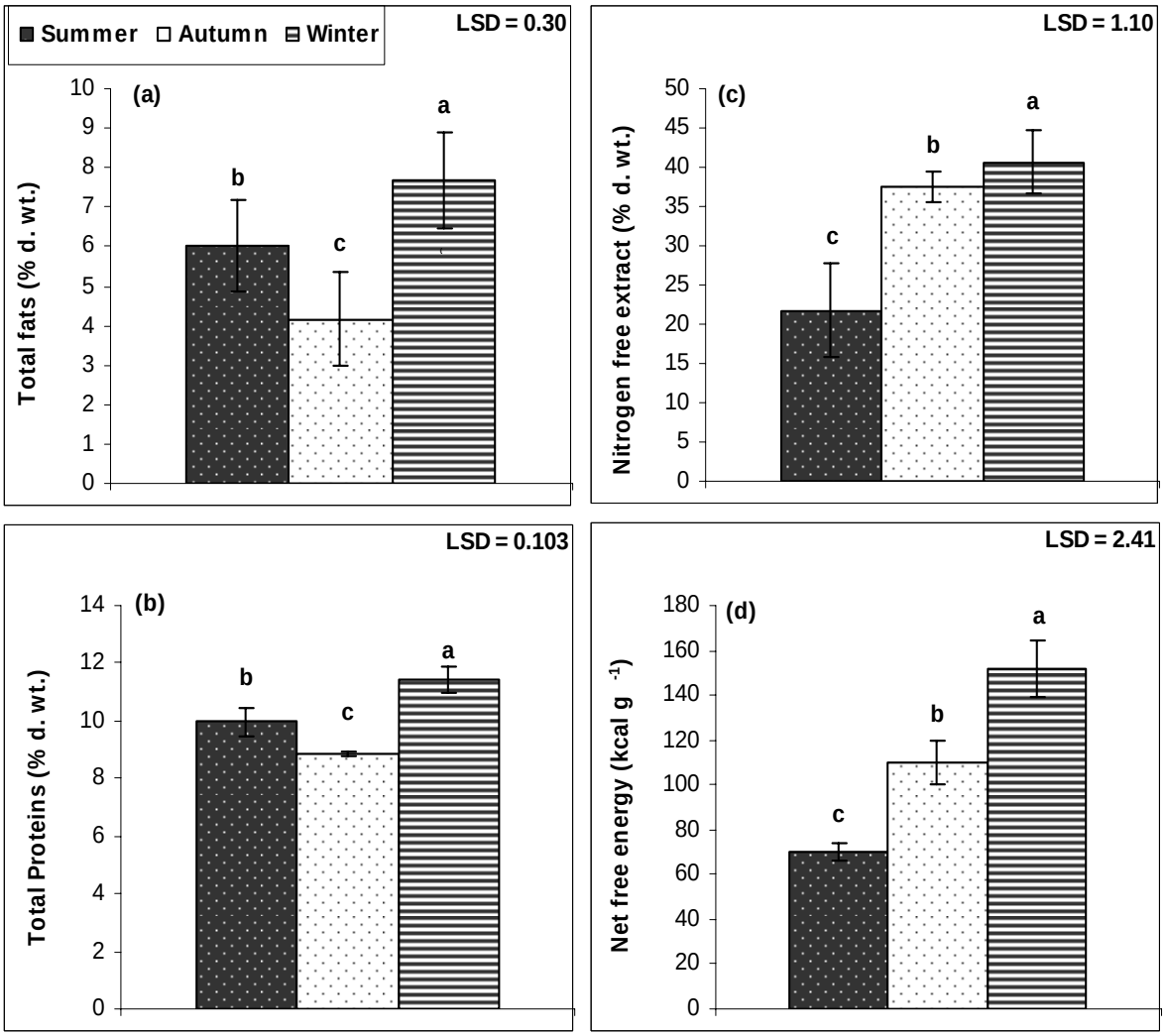

Fig. 2. Seasonal variation in (a) total fats, (b) total proteins (c) nitrogen free extract and (d) net free energy in the shoots of *M. longifolia* at Knotti Garden (d. wt. = dry weight).

Discussion

All the biochemical and medicinal ingredients in *Mentha longifolia* analyzed in this study varied significant during different seasons. Dry matter, total fibers, total minerals, and micro-nutrients (Zn, Fe, Mg and Cu) were the highest during the period of active growth in summer, declined during the period of less vigorous growth in autumn and were lowest at plant maturity in winter. Although the autumn season had the least effect on biochemical and medicinal ingredients, moisture contents, K and total alkaloids were highest in this season, lower in winter and the least in summer. The maximum values for total phenolics, flavonoids, fats, proteins, NFE and NFES, and macro-nutrients (Na, N, Ca and P) were recorded during the winter season. Such seasonal effects on biochemical and medicinal ingredients have earlier been observed in a number of studies in different plant species such as *Adhatoda vasica* (Pandita *et al*., 1983), *Mentha pulegium* (Stengele *et al.*, 1993), *Sargassum wightii* (Reeta, 1993), *Mentha spicata* (Kofidis *et al.*, 2004), *Toona sinensis* (Wang *et al.*, 2007), *Adiantum capillus-veneris* (Ahmad *et al.*, 2008), *Ocimum basilicum* (Hussain *et al.*, 2008) and *Ulva reticulata* (Shanrnugam & Palpandi, 2008).

The RDA ordination biplot, which shows the effect of seasons on the medicinally important ingredients of *M. longifolia* shoots, indicated that total minerals and dry matter were strongly associated with summer (Figs. 1 & 6). In addition, fibers and minerals such as Cu and Fe showed less association with this season. However, Zn and Mg contents were equally distributed between the summer and autumn seasons (Figs. 4 & 6). As *M. longifolia* sprouts during late spring and early summer (Underwood, 1981; Zavodnik, 1998), most of the minerals actively taken up are transported to the aerial parts to support active metabolism in shoots during the metabolically active period of growth (Nelson *et al.*, 1990; Tanner & Beevers, 2001). In addition, Cu, Fe, Zn and Mg are the components of a number of bio-molecules and also participate in a number of metabolic reactions as co-factors (Pessarakli, 2001; Taiz & Zeiger, 2006). Thus, their requirement in plants is relatively higher during the period of fast growth which occurs in summer season (Khan *et al.*, 2004; Ahmad *et al.*, 2008).

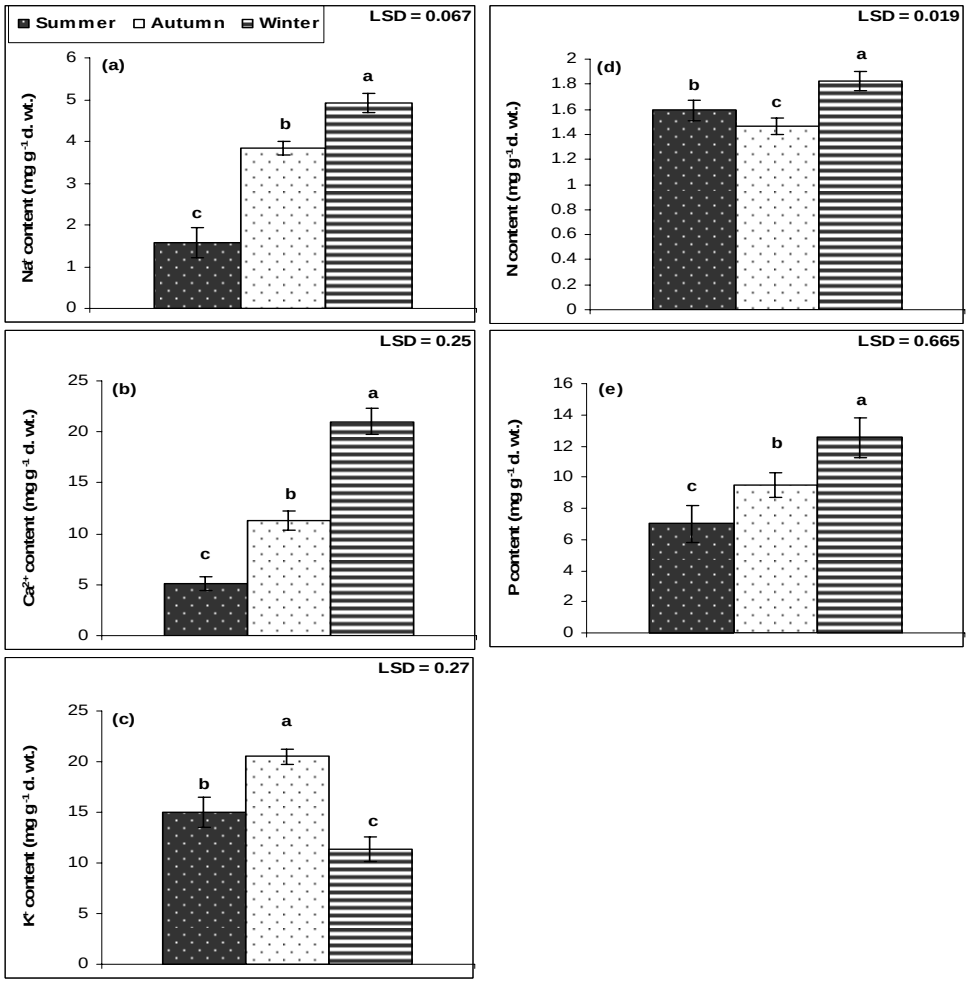

Fig. 3. Seasonal variation in (a) Na^{+}, (b) Ca^{2+} (c) K^{+}, (d) N and (e) P contents in the shoots of *M. longifolia* at Knotti Garden (d. wt. = dry weight).

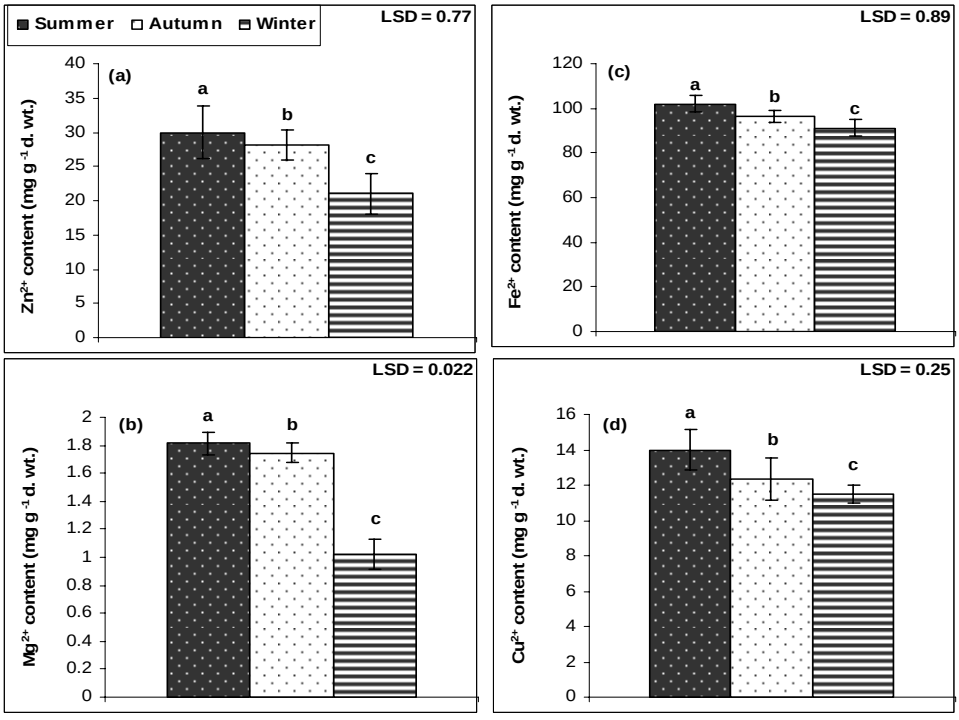

Fig. 4. Seasonal variation in (a) Zn^{2+}, (b) Mg^{2+} (c) Fe^{2+}, and (d) Cu^{2+} in the shoots of *M. longifolia* at Knotti Garden (d. wt. = dry weight).

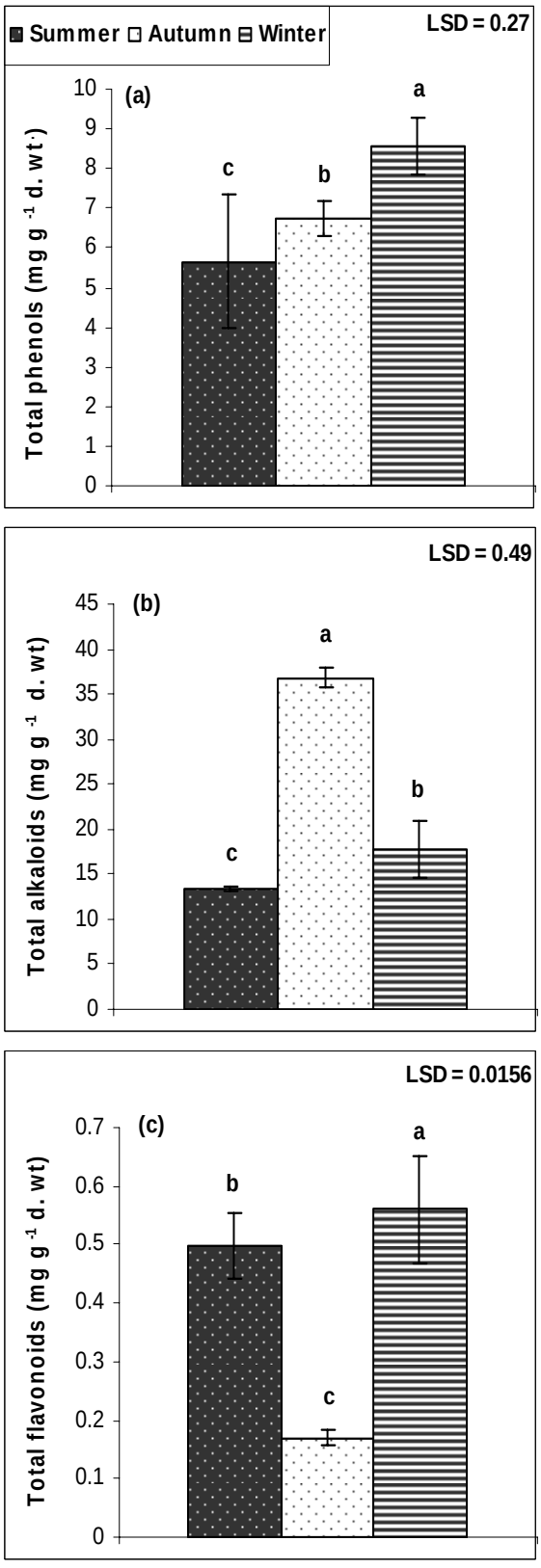

Fig. 5. Seasonal variation in (a) total phenols, (b) total alkaloids and (c) total flavonoids in the shoots of *M. longifolia* at Knotti Garden (d. wt. = dry weight).

Shoot moisture, alkaloids and K were strongly associated with the autumn season. However, nitrogen free extractable substances (NFES) were equally associated with the winter and autumn seasons (Figs. 2, 3 & 6). Higher moisture and K during winter might have been due to high moisture available in the soil as a result of frequent rainfall during this season (McDowell, 2003). In addition, the higher association of NFES with the autumn as compared to winter might have been due to nitrogen deficiency in soil and in plant tissues during this season. Furthermore, higher alkaloids might indicate some sort of nutritional stress in this season (Miranda-Ham *et al.*, 2007) or a complex interaction between soil and environment.

Total fats, proteins, phenolics, flavonoids, net free energy (NFE) and some minerals like N, P and Ca were associated with the winter season (Figs. 2, 3, 5 & 6). During winter, most of the herbs in the Soone Valley, including *Mentha longifolia*, complete their life cycle and start drying up. As fats and proteins are the end products of metabolic reactions in drying shoots, they are naturally higher at this stage (Akingbade *et al.*, 2001; Sahyun, 2008). Thus, as a result of high accumulation of fats and proteins, the shoots of *M. longifolia* contained maximum energy during the winter season (Ghimire *et al.*, 1999; Ahmad *et al.*, 2008). The higher concentration of phenolics and flavonoids during winter may have been due to low temperature stress as well as maturity of the plants (Buchanan *et al.*, 2000; Harborne & Williams 2000; Garmash, 2005). As N and P are integral components of a number of macro-molecules such as proteins and nucleic acids, their concentration could be higher in plants at maturity (Ghimire *et al.*, 1999; Hossain *et al.*, 2007; Ahmad *et al.*, 2008).

Conclusion

It is evident from this study that different biochemical attributes varied significantly during different seasons. Total fats, proteins, net free energy (NFE), nitrogen free extractable substances (NFES), macronutrients (Na, N, Ca and P) and phenolics increased, while dry matter, fibers, mineral, and micro-nutrients (Zn, Mg, Fe and Cu) generally decreased as plants matured through the autumn and winter. Although K and alkaloidal contents increased significantly in autumn, they decreased during the winter season. The reverse was true for flavonoids, which decreased in autumn but increased in winter. The results of this study suggest that the best harvesting season for flavonoid, phenolics, NFE, proteins and fats is winter. In addition, plants can be harvested during summer for total minerals and fibers. Although there was little correlation between autumn and medicinally active ingredients, this species can be harvested for total alkaloidal contents during this season.

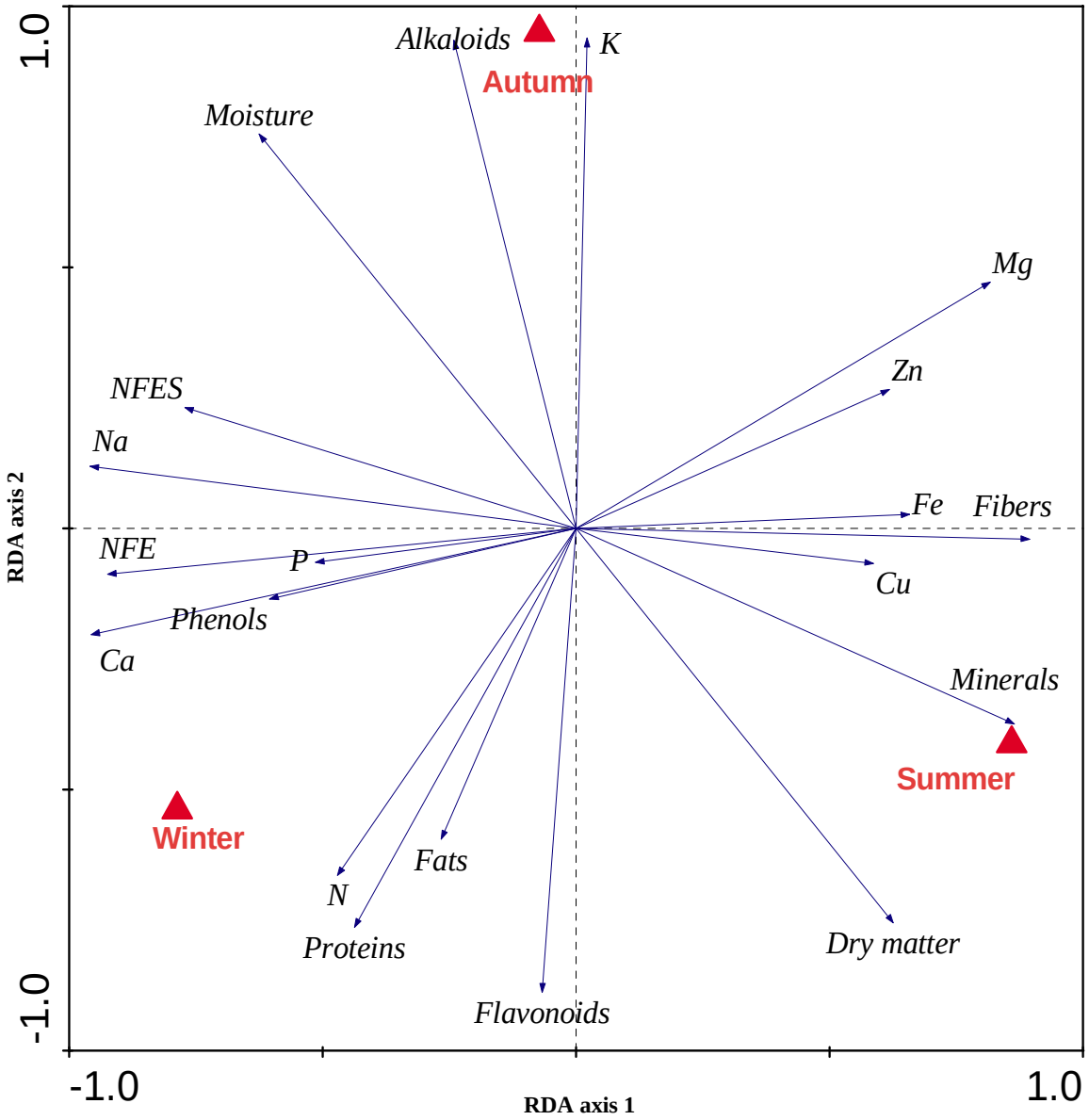

Fig. 6. *RDA* ordination biplot showing the effect of seasons on medicinal and biochemical attributes of *M. longifolia* shoots collected during different seasons from the Soone Valley of the Salt Range.

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