Botanica Orientalis – Journal of Plant Science (2010) **7:** 43–47 ISSN 1726-6858

http://www.cdbtu.edu.np/botanica-orientalis

Research

Variation in the active constituent contents in *Centella asiatica* grown in different habitats in Nepal

Anjana Devkota[1]*, Stefano Dall' Acqua[2], Pramod Kumar Jha[1] and Gabbriella Innocenti[2]

[1]Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal; [2]Department of Pharmaceutical Sciences, University of Padova, Padova, Italy

Abstract

Centella asiatica is an important medicinal plant of subtropical to tropical region. It grows widely, in different habitats. In Nepal, it is distributed at an altitudinal range of 96-2200 m above sea level. A comparative quantitative analysis of chemical constituents in *Centella asiatica* samples collected from three different habitats in Nepal was carried out by HPLC to evaluate the variability in the important constituents. There was marked variability in asiaticoside, asiatic acid and quercetin 3-*O*-glucuronide content among the samples collected from different habitats. Samples collected from open agricultural land showed the highest asiaticoside (1.91%), asiatic acid (0.13%) and quercetin 3-*O*-glucuronide (0.35%) content. Therefore, open land is preferable for plantation of this species for high yield of secondary metabolites.

Key-words: Asiaticoside, asiatic acid, *Centella asiatica*, HPLC, medicinal plants, quercetin 3-*O*-glucuronide.

Introduction

Centella asiatica (L.) Urban, also known as 'gotu kola' or 'Indian pennywort', is a tropical medicinal plant with a long history of therapeutic use, particularly in dermal disorders, venous insufficiency and microangiopathy. Reports from various places have revealed that *C. asiatica* has been used for wound healing (Shukla *et al*. 1999), memory improvement, bronchitis, asthma, dysentery, leucorrhoea, kidney trouble, anti-allergic and anticancer purposes, curing leucorrhoea and toxic fever (Kan 1986). Clinical trials have also shown that it can help those with chronic venous insufficiency (Brinkhaus *et al*. 2000). *Centella asiatic*a mainly contains asiatic acid, madecassic acid, terminolic acid, vanillic acid, succinic acid, asiaticoside, asiaticoside-B, madecassoside, asiaticodiglycoside. The main active components of the plant are believed to be triterpenoids. Several studies have revealed the triterpenoid derivatives of *Centella asiatica* using different techniques (Diallo *et al*. 1991; Du *et al*. 2004). A HPLC method was set for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products by Schaneberg *et al*. (2003). Recently, Devkota *et al*. (2010) obtained data about the variations in secondary metabolite in different geographical areas of Nepal selecting high producing triterpene plants for possible cultivation. In this study, we collected plant material from three different habitats, *viz*. open agricultural land, open grassland and shady grassland to analyze the influence of habitats on the eight main chemical constituents of *Centella asiatica*. We used an ELSD detector because of the poor UV absorption of the triterpene nucleus.

*Corresponding author, email address: devkotaa@gmail.com

Materials and Method

PLANT MATERIALS

Plant samples (aerial parts) ($n = 38$) of *Centella asiatica* were collected from different habitats in Nepal: (a) open grassland (where grazing pressure was high and vegetation was dense);

(b) partially shade grassland (where vegetation was dense, and grazing was prohibited) and (c) open agricultural land (moderately grazed open land, receiving full sunlight and with sparse vegetation). Samples were collected in April-May 2007 and shade dried.

COLLECTION AND ANALYSIS OF SOIL SAMPLES

Soil samples (n = 10), from each habitat, was collected from the root zone at the time of collection of plant samples. Then, samples were air dried and used for analysis. Soil organic carbon was determined by the Walkley Black rapid titration method and total N by micro-Kjeldahl method (Jackson 1958).

CHEMICALS AND REAGENTS

HPLC grade acetonitrile, methanol and formic acid were purchased from Carlo Erba Italy. HPLC grade water was prepared by filtering nanopure water through a 45 µm membrane filter (MilliQ).

REFERENCE SAMPLE

Asiaticoside, kaempferol, quercetin, rosmarinic and chicoric acids were purchased from Phytolab GmbH, Germany. Chlorogenic acid was purchased from Sigma Aldrich. Quercetin 3-O-glucuronide was purified from the extracts of *C. asiatica* as described by Satake *et al.* (2007).

PLANT SAMPLE PREPARATION

Approximately 100 mg of ground plant material (whole plant parts) was placed into a 15 ml falcon tube (screw capped polypropylene centrifuge tube) and extracted three times with 5.0 ml of methanol by sonication. The extract was centrifuged 5 minute in 3000 rpm and the supernatants were combined to a 25 ml volumetric flask by pipette, diluted to final volume with methanol and mixed thoroughly. All samples were filtered through a 0.45 µPTFE syringe filter before the injection in HPLC.

HPLC CONDITIONS

Instrumentation consisted of an Agilent 1100 series liquid chromatograph equipped with Agilent 1100 Diode Array (DAD) and SEDEX LT60 Evaporative Light Scattering Detectors (ELSD). An Agilent XDB-C-18 reverse phase column (25×4.6 mm, 4.6 µm) was used as stationary phase. The gradient elution program, with aqueous formic acid (0.1%) (A) and acetonitrile (B), was: 0-8.5 min, linear gradient from 12 to 26 % B; 8.5-11 min, isocratic conditions at 26 % B; 11-16 min, linear gradient from 26 to 40 % B; 16-45 min, linear gradient from 40 to 50 % B; 45-50 min, linear gradient from 50 to 100 % B. Flow rate was 1 mL/min and injection volume 20 µL. The ELSD detector temperature was 50°C, nitrogen pressure 2.2 bars, and the gain level 10 arbitrary units (a.u.). For the sample analyses, a gradient elution was used; using an eluent A: Acetonitrile, B: Methanol, C: water with 0.1% HCOOH. Gradient is presented in Table 1.

Table 1. Gradient scheme used in HPLC analyses.

Time (Minutes)	Solvent (ml)		
	Acetonitrile	Methanol	Water with 0.1% HCOOH
0	10	2	88
10	26	4	70
22	25	5	70
27	30	0	70

Calibration curves were obtained by preparing standard solutions, as listed in Table 2. Asiaticoside and asiatic acid were determined with the ELSD detector. Chlorogenic, chicoric and rosmarinic acids were determined with the DAD at 330 nm, and at 350 nm for kaempferol, quercetin and quercetin 3-*O*-glucuronide. HPLC chromatogram of the standard compounds is reported in Fig. 1.

DATA ANALYSIS

The data were analyzed to assess the difference in measured attributes among the habitats by one way analysis of variance (ANOVA) and the Duncan's homogeneity test using Statistical Package for Social Science, version 11.5 (SPSS 2002).

Results

HPLC analysis revealed marked variability in the analyzed bioactive components among the samples collected from different habitats (Table 3). Content of these phytochemicals varied greatly in different habitats depending upon the nature of chemical constituents. The amount of most of the analyzed chemical constituents was higher in open agricultural land than in other habitat types. However, the difference was statistically significant for asiaticoside, asiatic acid, quercetin 3-*O*-glucuronide, kaempferol and rosmarinic acid content ($p < 0.05$) (Table 3). Samples collected from open agricultural land showed the highest asiaticoside (1.91%), asiatic acid

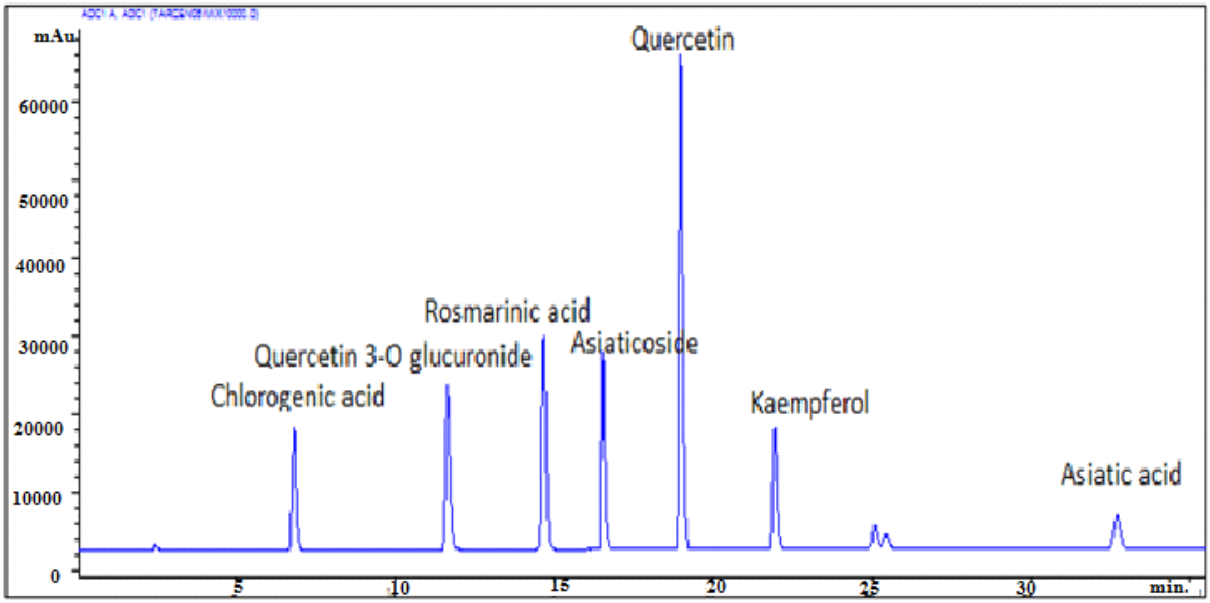

Figure 1. HPLC chromatogram of the standards used for the quantitative determination.

Table 2. Concentration ranges and calibration curves for the analyzed secondary metabolites.

Analyte	Concentration (µg/mL)	Regression curve*	R^2 (n = 6)	LOD (µg/mL)	LOQ (µg/mL)
Asiatic acid	4.250 - 100.00	Log y = 0.608 Log x – 1.880	0.9976	1.04	3.46
Asiaticoside	5.620 - 140.50	Log y = 0.588 Log x – 1.872	0.9989	1.02	3.40
Chicoric acid	0.732 - 73.17	y = 0.0127 x – 0.0886	0.9998	0.29	0.97
Chlorogenic acid	0.685 - 97.53	y = 0.0168 x – 0.2456	0.9989	0.40	1.33
Rosmarinic acid	0.766 - 113.00	y = 0.0147 x + 0.1534	0.9995	0.35	1.17
Quercetin	0.840 - 84.03	y = 0.0086 x + 0.9724	0.9982	0.21	0.70
Quercetin 3-O-glucuronide	1.300 - 130.00	y = 0.0393 x – 0.0249	0.9983	0.10	0.33
Kaempferol	0.660 - 65.97	y = 0.0136 x + 0.391	0.9997	0.19	0.63

*x = peak area; y = concentration of analyte (ìg/mL).

LOD = Limit of Detection, LOQ = Limit of Quantification.

Table 3. Phytochemical constituents of *Centella asiatica* from different habitats. For each parameter, significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, *á* = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Chemical constituents	Partially shade grassland (n=12)	Open grassland (n=12)	Open agricultural land (n=14)	Mean	F value	P value
Asiaticoside	$1.41^{a} \pm 1.10$	$1.71^{b} \pm 1.03$	$1.91^{b} \pm 1.03$	1.68 ± 1.60	10.334	0.030
Asiatic acid	$0.08^{a} \pm 0.01$	$0.07^{a} \pm 0.02$	$0.13^{b} \pm 0.11$	0.09 ± 0.02	0.604	0.012
Chicoric acid	$0.05^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	0.05 ± 0.10	0.603	0.321
Chlorogenic acid	$0.24^{a} \pm 0.18$	$0.23^{a} \pm 0.12$	$0.26^{a} \pm 0.08$	0.25 ± 0.13	0.117	0.190
Quercetin	$0.35^{a} \pm 0.10$	$0.38^{b} \pm 0.02$	$0.38^{b} \pm 0.00$	0.37 ± 0.06	1.154	0.198
Quercetin 3-*O*-glucuronide	$0.17^{a} \pm 0.00$	$0.28^{b} \pm 0.01$	$0.35^{c} \pm 0.01$	0.25 ± 0.01	17.24	0.025
Kaempferol	$0.03^{a} \pm 0.11$	$0.35^{a} \pm 0.10$	$0.39^{b} \pm 0.06$	0.36 ± 0.09	7.301	0.042
Rosmarinic acid	$0.18^{a} \pm 0.07$	$0.16^{a} \pm 0.08$	$0.18^{b} \pm 0.17$	0.15 ± 0.12	1.301	0.032

Table 4. Nutrient content in soils from different habitats. For each parameter, significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, $\acute{a} = 0.05$). F and P values were obtained by one way ANOVA.

Habitats	Soil Nitrogen (%)	Soil Organic Carbon (%)	Soil Organic Matter (%)
Partially shade grassland	$0.24^{a} \pm 0.12$	$2.34^{a} \pm 0.98$	$4.23^{a} \pm 1.73$
Open grassland	$0.13^{b} \pm 0.05$	$1.16^{b} \pm 0.51$	$2.03^{b} \pm 0.89$
Open Agricultural Land	$0.13^{b} \pm 0.23$	$1.52^{b} \pm 0.47$	$2.58^{b} \pm 0.82$
F value	16.72	9.23	22.13
P value	<0.001	<0.001	<0.001

(0.13%) and quercetin 3-*O*-glucuronide (0.35%) content. Asiaticoside was the most dominant constituent (mean 1.68% dw); its value ranged from 1.41% in shady grassland to 1.91% in open agricultural land (Table 3). The content of chicoric acid, chlorogenic acid and quercetin did not differ significantly among the habitats.

There was significant difference in nutrients in soil collected from different habitat (Table 4). Soil N was higher in open agricultural land than in other sites.

Discussion

Present study showed that habitat factor may impose significant impact on accumulation of important bioactive components in plants. Significantly higher amount of phytochemical constituents was measured in samples collected from open agricultural land and least from shady grassland. The plants growing on open agricultural land are possibly under stress due to direct sunlight and less availability of moisture. Perhaps due to increased solar radiation and temperature, the plants produced more secondary compounds in relation to the adaptation mechanism. Odabas *et al.* (2009) hypothesized that the high photosynthetic activity under high light intensity resulted on increased amount of carbon assimilation and enhanced the concentration of carbon-rich secondary metabolites in leaf tissues. In this study, soil from open agricultural land contained relatively low nutrient (C and N) as compared to shady habitat (Table 4). Nutrient stress generally reduces growth more than it reduces photosynthesis per second (McKey 1979) and thus, it has been argued that the expected surplus of carbon can lead to an accumulation of carbon-based secondary substances under such circumstances (Bryant *et al.* 1983). This might be the reason for having low amount of secondary metabolites in shady grassland with relatively high soil nutrient contents.

Significant difference in contents of active constituent have been observed in samples of *C. asiatica* originating from different countries, such as India and Madagascar (Das and Mallick 1991; Rouillard-Guellec *et al.* 1997). Comparative study by Das and Mallick (1991), in 10 ecotypes of *Centella asiatica* from different regions of India, showed a correlation between genomic diversity and asiaticoside content. In present study, mean asiaticoside content in samples of *Centella asiatica* was 1.68% (dw). It has been shown that leaves of *C. asiatica* from Nepal contain 4 to 10 time's higher concentration of asiaticoside than those from India (Rouillard-Guellec *et al.* 1997). Low quantity of asiatic acid and chicoric acids were recorded in all analyzed samples (Table 3). Foo and Porter (1980) have reported that the compounds with lower molecular weight are usually present in plant tissue in relatively low concentrations compared to that of larger polymers. Generally, all *C. asiatica* samples showed relatively higher amount of asiaticoside than asiatic acid. This is in accordance with large amount of triterpene glycosides and trace of triterpenic acids from plants of Thailand, Costa Rica and Bahamas (Booncong 1989). However, high asiatic acid content was reported in *C. asiatica* of Malaysia (Pick Kiong 2004). The observation in this study is in agreement with the statement by many researchers that *C. asiatica* collected from different locations produced different amount of triterpenes. Apart from the environment, climate and soil condition, the method of extraction could also be a contributing factor for the diverse compounds in *C. asiatica* from various locations (Booncong 1989).

Conclusion

Different habitats have significant effect on accumulation of active phytochemicals in *C. asiatica*. Open land is preferable for plantation of this species for high yield of secondary metabolites, especially the marker compound (asiaticoside).

Acknowledgements

The first author is grateful to Padova University (Italy) for giving opportunity to work in the laboratory of Department of Pharmaceutical Sciences, Padova, Italy under Tribhuvan University and Padova University exchange program.

References

Booncong P. 1989. *A Pharmacognostic and Taxonomic Study of* Centella asiatica (Apiaceae). PhD Thesis, Miami University, Ohio, USA.

Brinkhaus B., Linder M., Schuppan D. and Hahn E.G. 2000. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine* 7: 427–448.

Bryant J.P., Chapin F.S. and Klein D.R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40: 357–368.

Das A. and Mallick R. 1991. Correlation between genomic diversity and asiaticoside content in *Centella asiatica* (L.) Urban. *Botanical Bulletin of Academia Sinica* 32:1–8.

Devkota A., Dall'Acqua S., Comai S., Innocenti G. and Jha P.K. 2010. *Centella asiatica* (L.) Urban from Nepal: quali-quantitative analysis of samples from several sites, and selection of high terpene containing populations for cultivation. *Biochemical Systematics and Ecology* 38: 12–22.

Diallo B.,Vanhaelenfastre R. and Van-Heusen M. 1991. Direct coupling of high speed counter current chromatography to thin layer chromatography – application to the separation of asiaticoside and madecassoside from *Centella asiatica*. *Journal of Chromatography* 558: 446–450.

Du Q., Jerz G., Chen P. and Winterhalter P. 2001. Preparation of ursane triterpenoids from *Centella asiatica* using high speed counter current chromatography with step – gradient elution. *Journal of Liquid Chromatography and Related Technology* 27: 2004–2007.

Foo L.Y. and Porter L. 1980. The phytochemistry of proanthocyanidin polymers. *Journal of Phytochemistry* 19: 1747–1752.

Jackson M.L. 1958. *Soil Chemical Analysis*. Prentice Hall, NJ, USA.

Kan W.S. 1986. *Pharmaceutical Botany*. National Research Institute of Chinese Medicine, Taipei, Taiwan.

McKey D. 1979. The distribution of secondary compounds within plants. In: *Herbivores: Their Interaction with Secondary Plant Metabolites* (G.A. Rosenthal and D.H. Janzen, eds.), pp. 55–133. Academic Press, New York, USA.

Odabas M.S., Jolita R., Necdet C., Valdimaras J., Liudas I and Cüneyt Ç. 2009. The quantitative effects of temperature and light intensity on hyperforin and hypericins accumulation in *Hypericum perforatum*. *Journal of Medicinal Plants Research* 3(7): 519–525.

Pick Kiong A.L. 2004. Triterpene production in *Centella asiatica* (L.) Urban (pegaga) callus and cell suspension cultures. Ph.D. Thesis, School of Graduate Studies, Putra University, Malaysia.

Rouillard-Guellec F., Robin J.R., Ratsimamanga A.R., Ratsimamanga S. and Rasaoanaivo P. 1997. Comparative study of *Centella asiatica* of Madagascar origin and Indian origin. *Acta Botanica Gallica* 144: 489–493.

Schaneberg B.T., Mikell J.R., Bedir E. and Khan A. 2003. An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. *Pharmazie* 58: 381–384.

Satake T., Kamiya K., An Y., Oishi N., Taka T. and Yamamoto J. 2007. The anti-thrombotic active constituents from *Centella asiatica*. *Biological and Pharmaceutical Bulletin* 30: 935–940.

Shukla A., Rasik A.M. and Dhavan B.N. 1999. Asiaticoside induced elevation of antioxidant levels in healing wounds. *Phytotherapy Research* 13: 50–54.

SPSS. 2000. *Statistical Package for Social Sciences (SPSS), Version 11.5*. SPSS Inc., USA.