Journal of Applied Pharmaceutical Science Vol. 4 (02), pp. 043-047, February, 2014
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2014.40207
ISSN 2231-3354

Screening of proper leaf size in *Centella asiatica* for antioxidant potential and separation of phenolics using RP-HPLC

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ARTICLE INFO

Article history:
Received on: 02/12/2013
Revised on: 22/12/2013
Accepted on: 20/02/2014
Available online: 27/02/2014

Key words:
Centella asiatica, growth, chlorophyll content, antioxidant activity and phenolics.

ABSTRACT

Centella asiatica is a valuable medicinal plant with abundant amount of pharmaceutically useful compounds; however, it is important to know the proper physiological age for drug formulation. In the present study, smaller to larger thirteen groups of *Centella asiatica* leaves were evaluated for growth, chlorophyll content and antioxidant activity. The growth was measured in the terms of leaf area, fresh and dry weights and water content. Maximum antioxidant activity was found in third size group of the leaf which was further analyzed for phenolics in RP-HPLC. The probable role of these phenols in antioxidant activities is discussed.

INTRODUCTION

Ever since civilization, use of plants in various health problems is recognized worldwide. Plants have ability to synthesize a wide variety of biologically functional compounds, e.g. primary and secondary metabolites. Many of these phytochemicals (12,000 such compounds) have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat human diseases (Tapsell *et al.*, 2006). However, the effectiveness of the aurvedic formulation is questioned many times or it has reported slow responses. Applications of these compounds for pharmaceutical preparations require a proper selection of plant materials. The desired compound may not have required concentration throughout the growth and development time of the plant part use. Most of the phytochemical studies concentrated on leaves and the constituents vary depending upon the geographical distribution (Chong and Aziz, 2011). Therefore it is imperative to investigate the appropriate age of the plant for better and effective drug formulation. *Centella asiatica* is called Brahmi belongs to family Apiaceae (Umbelliferae) is being used as a natural source of medicine for long time.

It is extensively used in wound healing, cleansing for skin problem and digestive disorders (Chevallier, 2001) and effective in treatment of stomach ulcers, mental fatigue, diarrhea, epilepsy, hepatitis, syphilis and asthma. *C. asiatica* contains chemicals known as triterpenoids that appear to speed wound healing, boost antioxidants at the wound site, strengthen the skin and increase blood supply to the wounded area (Goldstein and Goldstein, 2012). The most used parts for medicinal purposes are dried whole plant, leaves and stems. *C. asiatica* is a rich source of amino acids, flavonoids, terpenoids, essential oils, alkaloids etc. (Puttarak and Panichayupakaranant, 2012).

In this study an attempt is made to evaluate the antioxidant potential of the leaf tissue in *C. asiatica* is and further analyzed for the phenolic separation using HPLC. The main objective of the study was to understand (a) at which stage the plant have maximum antioxidant activities and (b) the probable phenolics that may have key role in antioxidant activity of the leaf.

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MATERIALS AND METHOD

Collection of plant material

Centella asiatica leaves were collected from the Botanical Garden of Saurashtra University campus. It was separated in 13 groups as per their smaller to larger size (Plate 1).

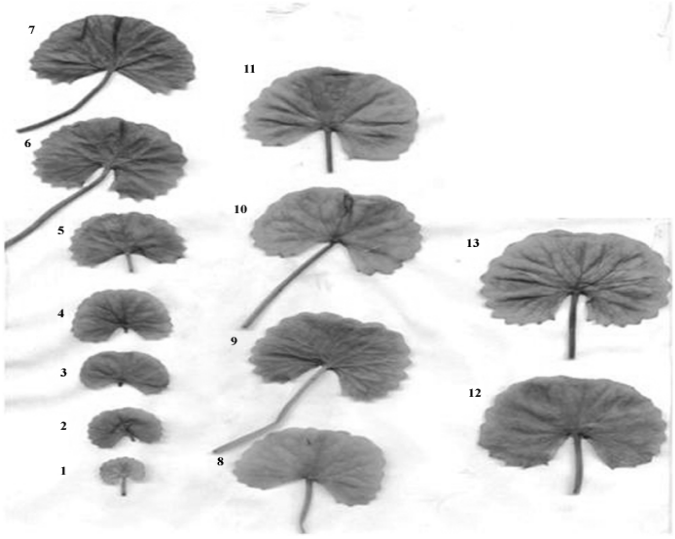

Plate. 1: different size of *Centella asicatica* leaves.

Identification and confirmation of the sample

The plant was authenticated by Prof. Vrinda Thaker, Department of Biosciences, Saurashtra University and Voucher specimen (CPBGE -109) is deposited in the herbarium of same Department. For further identification, *rbcL* barcode sequence was generated using the amplified PCR product of plant sample on the ABI 3130 Genetic Analyzer and the sequence was deposited to the NCBI (Gene bank database, USA). The accession number obtained was JX125090 of the *rbcL* sequence.

Extraction and preparation of extract

Different size of leaves were collected and homogenized with a mortar and pestle containing 10 ml of 80% methanol. The samples were centrifuged at 10,000 g for 10 min and supernatant was collected. The pellet was washed with 80% methanol till it was free from the chlorophyll pigments. The supernatants were pooled and used for the estimation of chlorophylls and antioxidant activity.

Growth analysis

Growth was measured in the terms of fresh weight, dry weight, water content and leaf area. For the measurement of fresh and dry weights, freshly harvested smaller to larger sized of leaves were taken. Freshly separated leaves were weighed before and after oven drying to a constant weight at 65 °C for 48 hours to obtain the data on fresh and dry weights. Water content of each stage was determined by difference in fresh and dry weights. For each data point, three replicates were taken and mean value of dry weight and water content was calculated.

Leaf area and chlorophyll measurements

Leaf area was measured by leaf area meter software Muchhadia and Thaker (2006). Chlorophyll content was determined according to Arnon (1949) using spectrophotometer. Data for chlorophyll a and b were recorded at 645 and 663 nm.

DPPH free radical scavenging assay

Radical scavenging assay was determined according to Valazquez et al, (2003) using DPPH (1,1-diphenyl-2- picryl hydrezyl) free radical scavenging assay and color change from purple to yellow, which is measured on microplate reader (µ Quant, Bio Teak, USA).

RP-HPLC analysis

Reverse phase chromatography analyses were carried out under gradient condition using a Luna C-18 column (250 mm × 4.6 mm) packed with 5µm diameter particles. The mobile phase was solvent A (0.2% H_3PO_4 in milli q) and solvent B (0.2% H_3PO_4 in methanol). The gradient program was started with 90% A and 10% B; 0-1 min, 25% A and 75% B; 1-65 min, 0% A and 100% B; 66-95 min. 100% A and 0% B; 96-100 min., 90%A and 100% B; 100-120 min. respectively. The flow rate was 1ml/min. and the injection volume was 20 µl. The temperature of column was maintained at 28 °C. The detection wavelength of PDA was set at 290 nm. Prior to each run, the HPLC-PDA system was allowed to warm and the baseline was monitored until it was stable before sample analysis. Data were obtained by Shimadzu class LC solution software and the results were obtained by comparison with standard. Results are mean values from three replicates analysis of the same sample.

Statistical analysis

Experimental data was analyzed using analysis of variance (ANOVA) and significant difference among means from triplicate analyses at ($P<0.05$) were determined. All estimations were done in triplicates and mean values with standard deviations was calculated.

RESULTS AND DISCUSSION

In the present study, healthy leaves were collected for the analysis of growth parameters and antioxidant activities. The chlorophyll contents also evaluated from the each stage of the leaves and their statistical significance was worked out to know the relationship with growth and the antioxidant potential. Plant growth was measured as fresh wt. (gm/leaf), dry wt. (gm/leaf), water content (gm/leaf) and leaf area (mm^2). It was observed that the fresh and dry weights of the leaf increased gradually with the size of the leaf. Although the maximum value of the growth data like fresh and dry weight were observed at the last stage i.e. 13^{th}, the values from 7-10 showed very minor difference. However, gradually increased with stages of leaves was apparent in both fresh and dry weights (fig. 1A and 1B). Similarly, water content was also very high at the later stage of the leaf.

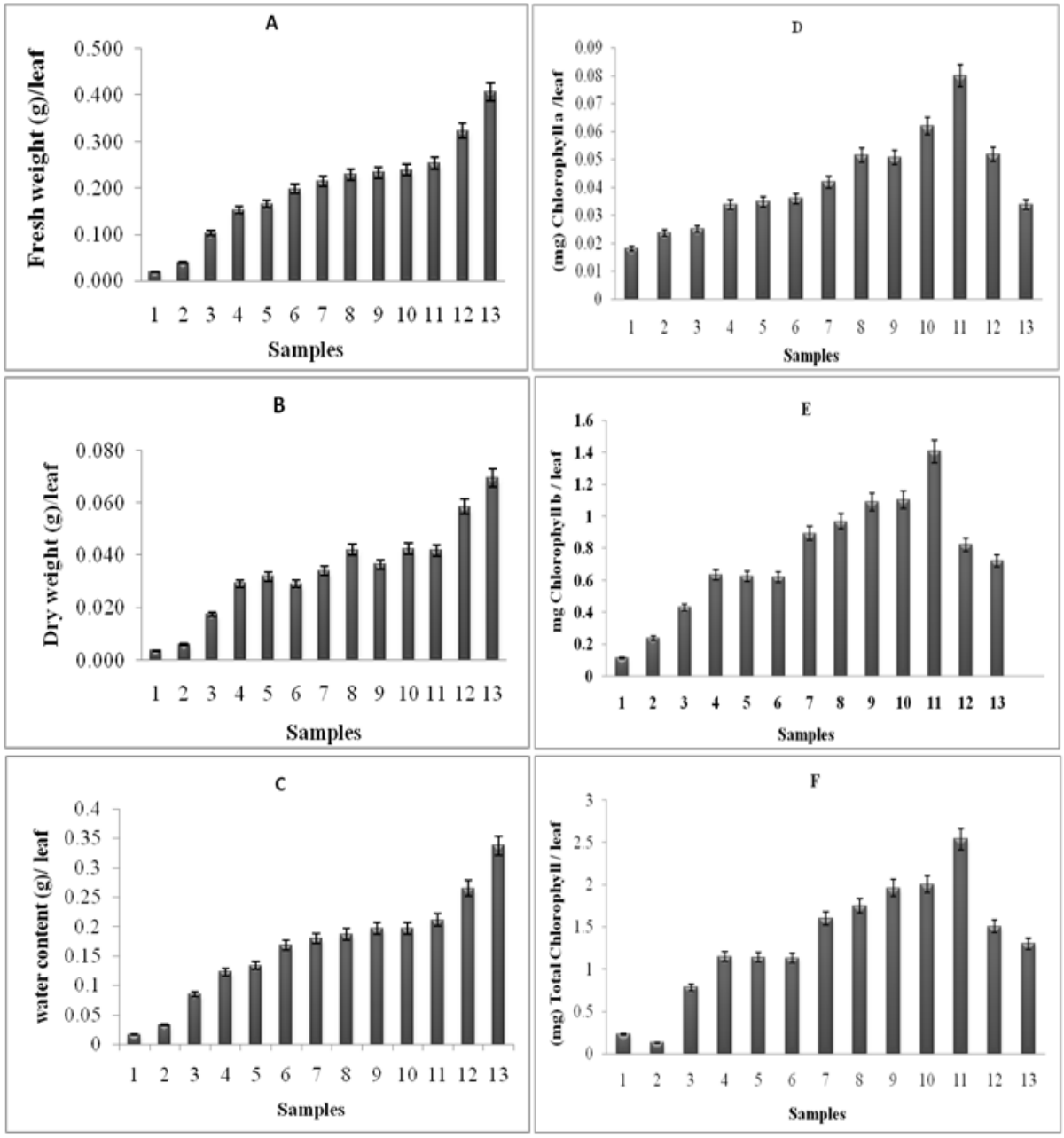

Fig. 1: Changes in (A) Fresh Weight (B) Dry Weight (C) Water Content (D) Chlorophyll a (E) Chlorophyll b (F) Total Chlorophyll content in leaf of *Centella asiatica* aginst size group.

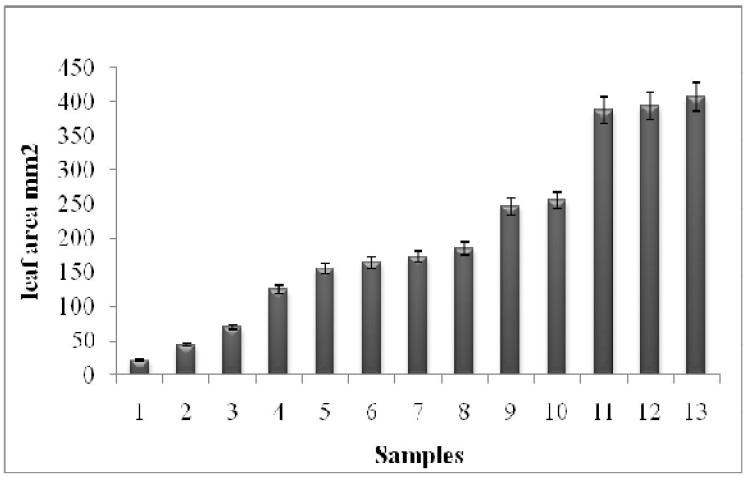

Fig. 2: Leaf area measurement.

The stabilization of value was evident at 7-11th stages of leaf development (fig. 1C). The changes in leaf area were also increased from stage one to 13th and maximum value was at stage 13th (fig. 2). The correlation worked out with the various growth parameters showed that leaf area showed more correlation with dry weight of the leaf (P< 0.001) followed by water content and fresh weight. Similarly, the data on chlorophyll content was also increased with the leaf age (fig. 1D, 1E) The total chlorophyll was increased with increase in leaf size which deceased at the latter two stages due to the senescence (fig. 1F). The relationship of chlorophyll content and growth parameters are reported in Table-1. It was observed that all parameters showed significant relationship with the chlorophyll content. It is assume that the growth and chlorophyll content relationship maintain constant irrespective of geographical distribution. This may help in designing of Aurvedic formulation for the effective drug preparation. Among natural substances, phenolic compounds and pigments, such as chlorophylls and carotenoids are the major candidate for antioxidant property. All the phenolic classes have the structural characteristics of free radical scavengers and have potential as food antioxidants (Bandoniene and Murkovic, 2002). While, chlorophylls and carotenoids are the pigments show the antioxidant properties with different levels (Chen and Chan, 1996; Ursula *et al.,* 2005). In the present study, the changes in antioxidant activities are reported in (fig. 3). The antioxidant activity gradually increase in 1st, 2nd and 3rd stages of leaves and the maximum antioxidant activity present in third stage of leaf (1763.7mg/F.W. leaf). It is interesting to note that young leaf have more antioxidant potential as compared to the older ones.

These activities showed very high positive correlation with chlorophyll a (Table-2). Antioxidant activities also showed relation with dry weight and leaf area (P< 0.005 and 0.05, respectively). The relationship with fresh weight and leaf area was relatively poor. It is interesting to work out the probable phenolic compounds present at the stage when remarkable antioxidant activities were evident. Therefore in the present study, the stage third was further analyzed for the detail separation of phenolic compounds.

In RP-HPLC analysis showed that gallic acid, gentisic acid, anisic acid, ferulic acid, coumarin, salicylic acid, quercetin, tannic acid (Table 2) was separated in third stage of leaf (Figure 4). These phenols may have a crucial role in antioxidant properties of the studied material. All the phenols matched the retention time of standard library of the instrument used. They are compounds having an aromatic ring with one or more hydroxyl groups and functional derivatives (Shahidi and Naczk, 2003) . All the phenolic classes have the structural characteristics of free radical scavengers and have potential as food antioxidants (Bandoniene and Murkovic, 2002).

Flavonoid compounds were present in aqueous extract of *C. asiatica*, showed highest antioxidant property (Pittella *et al*., 2009). To study the antioxidant properties and phenolic compounds present in *C. asiatica,* the optimum brewing procedure was studied to use as herbal teas (Ariffin *et al.,* 2011). From the results it is concluded that the younger leaves are the better source for the antioxidant activities. The identification of different Phenolic compounds from *Centella asiatica* will be used for the pharmacotherpeutic value.

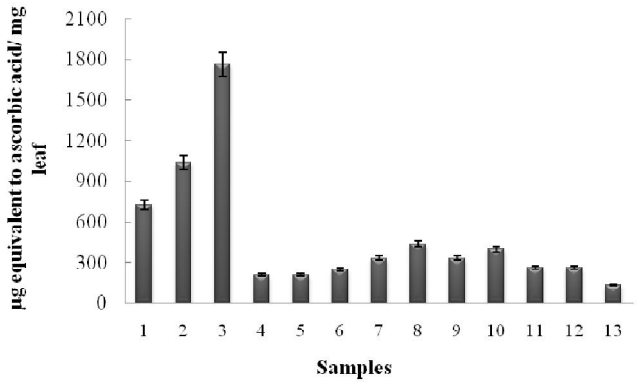

Fig. 3: Changes in antioxidant activity of *Centella asiatica* agisnt size group

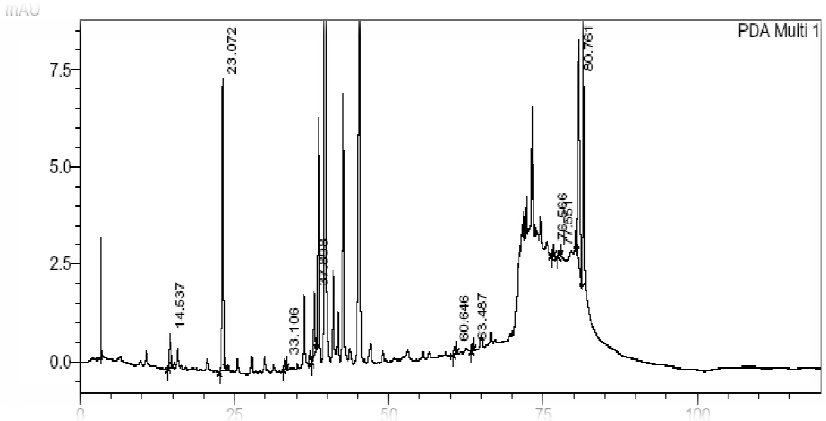

Fig. 4: Chromatogram of *Centella asiatica* leaf extract at the time of maximum antioxidant activities.

Table. 1: Result of single factor ANOVA between growth parameters and chlorophyll contents; growth parameters & antioxidant activity. The value shows p values, df 1, 22.

Parameter	Dry wt	Water content	Leaf area	Anti oxidant activity	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Fresh wt	0.001	0.385	0.923	0.079	0.0001	0.0001	0.0001
Dry wt		0.0001	0.0001	0.004	0.001	0.001	0.0001
Water content	0.0001		0.387	0.046	0.0001	0.0001	0.0001
Leaf area	0.0001	0.387		0.089	0.0001	0.0001	0.0001
Anti oxidant activity	0.004	0.046	0.089		0.0001	0.002	0.002
Chlorophyll a	0.0001	0.0001	0.0001	0.0001		0.013	0.012
Chlorophyll b	0.0001	0.0001	0.0001	0.002	0.002		1
Total Chlorophyll	0.0001	0.0001	0.0001	0.0024	0.013	1	

Table. 2: The retention time for different phenol detected in *Centella asiatica* leaf.

No.	Phenols	Retention time	No.	Phenols	Retention time
1	Gallic acid	14.53	5	Coumarin	60.64
2	Gentisic acid	23.07	6	Salicylic acid	64.98
3	Anisic acid	33.1	7	Quercetin	77.55
4	Ferulic acid	37.89	8	Tannic acid	81.59

ACKNOWLEDGEMENTS

The authors are thankful to Centre for Advanced Studies in Plant Biotechnology & Genetic Engineering, State Government for providing lab facilities, Gujarat, India

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How to cite this article:

Ritisha N. Tatmiya, Kiran S. Chudasama, Vibhuti M. Jhala and Vrinda S. Thaker. Screening of proper leaf size in *Centella asiatica* for antioxidant potential and separation of phenolics using RP-HPLC. J App Pharm Sci, 2014; 4 (02): 043-047.