South African Journal of Botany 112 (2017) 237–245

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Phytochemical and pharmacological evaluation of organic and non-organic cultivated nutritional *Centella asiatica* collected after different time intervals of harvesting

Rupa D. Bhattacharya, Komal M. Parmar, Prakash R. Itankar, Satyendra K. Prasad *

Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India

ARTICLE INFO

Article history:
Received 13 March 2017
Received in revised form 30 May 2017
Accepted 5 June 2017
Available online xxxx

Edited by V Steenkamp

Keywords:
Centella asiatica
Nootropic activity
Organic farming
Asiatic acid
Harvesting
Phytochemical standardization

ABSTRACT

The aim of the present study was to perform a comparative phytochemical and pharmacological evaluation of organically and non-organically cultivated nutritional *Centella asiatica* collected at different time intervals of harvesting. The fertilizers and pesticides applied in case of organic cultivation included combination of medicinal plants along with other natural products and the harvesting was carried out after 1st, 2nd and 3rd month of cultivation. From all the physicochemical parameters evaluated, the leaves collected after third month of harvesting showed higher ash values suggesting higher contents of sand or earthy matter along with higher moister content. The results from the phytochemical evaluations revealed that, the samples collected from first harvesting showed higher quantities of phytoconstituents. This was also confirmed through chromatographic studies using HPTLC showing higher content of Asiatic acid in samples collected after first month of harvesting, which was more pronounced in case of organic cultivation. The pharmacological evaluation included comparative nootropic activity of different samples of organic and nonorganic *C. asiatica*. The results depicted a significant ($p < 0.05$) reduction in escape latency of mice treated with 100 mg/kg, p.o. of organic and nonorganic cultivated *C. asiatica* collected after first month of harvesting and also showed a significant increase in the spatial working memory, which included acquisition and retrieval trials. Further, the two samples also revealed significant inhibition in AChE activity as observed in different brain region i.e. hippocampus, prefrontal cortex and amygdale, where organically cultivated *C. asiatica* was found to be more effective. Thus, from the overall observation, it may be concluded that, the organically cultivated *C. asiatica*, if collected after first month of cultivation shows the best memory enhancing activity as compared to other time interval of harvesting.

1. Introduction

Over two millennia ago, the father of medicine, Hippocrates, mentioned about 400 medicinal plants and advocated "Let food be your medicine and medicine be your food". Medicinal usage may constitute the most common human use of biodiversity (Anonymous, 2002). Nowadays, many consumers prefer organic to non-organic foods because they are perceived healthier lifestyle (Lohr, 2001). In general, organic practices are thought to reduce the risk of plant infection by pathogens. However, there is some evidence that the reduced use of fungicides may lead to a greater contamination by mycotoxins in organic food (Juan et al., 2007). Organic agriculture means a farming system which produces a healthier and quality products, improvement of the quality of life, preservation of the organic diversity, improvement of the soil structure and the balance of soil inhabiting microorganisms; without any application of synthetic product. Due to the Biodiversity Act 2002, the demand of organically cultivated medicinally plant is raised. Therefore, presently there is a huge turn around in organic cultivation of medicinal plants. Various studies have revealed that organically cultivated medicinal plant such as *Oryza sativa* (Singh et al., 1996) has shown to possess a higher bioactive secondary metabolite as compared to conventionally cultivated medicinal plant.

There has been a substantial increase in the global and national demand of medicinal plants, since last two decades. As consequences, the safety and quality of herbal medicines have become increasingly

Abbreviations: AChE, acetyl cholinesterase; AMY, amygdale; CCA, Control *C. asiatica* collected from wild habitat; CMC, Carboxymethyl cellulose; HIP, Hippocampus; HPTLC, High Performance Thin Layer Chromatography; MWM, Morris Water-Maze; NOCA1, Non-organic *C. asiatica* collected after 1st month of harvesting; NOCA2, Non-organic *C. asiatica* collected after 2nd month of harvesting; NOCA3, Non-organic *C. asiatica* collected after 3rd month of harvesting; OCA1, Organic *C. asiatica* collected after 1st month of harvesting; OCA2, Organic *C. asiatica* collected after 2nd month of harvesting; OCA3, Organic *C. asiatica* collected after 3rd month of harvesting; OECD, Organization for Economic Co-operation and Development; PFC, prefrontal cortex; R_f, Retention factor; SRM, spatial reference memory; SWM, spatial working memory; TBA, tertiary-butyl alcohol.

* Corresponding author.
E-mail address: skprasad.itbhu@gmail.com (S.K. Prasad).

http://dx.doi.org/10.1016/j.sajb.2017.06.003
0254-6299/

major concern both in health and pharmaceutical sector (Ekor, 2014). Crude drug quality, as quoted by Acharya Charaka depends upon major factor like Dasha (habitat), kala (time), Guna (property), and Bhajnana samapta (cultivation area). Kala is one of the major factors that influence the potency of medicinal plants. Avastha (phonological growth) can also be related to the immature stage to maturing stage of medicinal plants as stated by Charaka and also has a great impact on the quantitative and qualitative aspect of drug. The basic point of consideration like suitable time of collection of plant, knowledge of storage techniques of crude drug plays an inevitable role in quality and safety of the finished medicinal plant product (Ranade and Acharya, 2015). Plant diversity has a considerable importance as a source of pharmaceutically active substances. Environmental conditions affect, not only plant growth but also influence secondary metabolites productions. The medicinal plants show a marked variation in active principles during different seasons and time of harvesting; as these have been widely attributed to variations in environmental variables such as temperature and rainfall (Soni et al., 2015).

Centella asiatica Linn (Apiaceae) is native to Southeast Asian countries such as India, Sri Lanka, China, Indonesia, Malaysia as well as South Africa and Madagascar. *C. asiatica* is known to be consumed as leafy green vegetable in Sri Lanka and Philippines (Orhan, 2012). The leaves are used for preparing a drink and also eaten in raw form as salads or cold rolls in Vietnam, India, and Thailand due to its high amounts of medicinally important triterpenoids and beneficial carotenoids (Orhan et al., 2013; Chandrika and Prasad Kumarab, 2015). *C. asiatica*, has been reported to show a wide range of biological activities desired for human health such as wound healing (Suguna et al., 1996), anti-inflammatory (Somchit et al., 2004), anti-psoriatic (Sampson et al., 2001) anti-ulcer (Cheng et al., 2004), hepatoprotecive (Pingale, 2008), anticonvulsant (Sudha et al., 2002), sedative (Wijeweera et al., 2006), immunostimulant (Wang et al., 2003), cytotoxic and antitumor (Lee et al., 2002; Bunpo et al., 2004).

The incidence of age related dementia and brain disorders is dramatically on the rise as life expectancy increases. Nootropic (noos—mind, tropein—towards) drugs belong to the category of psychotropic agents with selective facilitatory effect on intellectual performance, learning and memory (Giurgea, 1973). Alzheimer's disease (AD) is a progressive neurodegenerative disorder and causes significant dementia in elderly people (Firuzi and Pratic, 2006). In traditional practices, medicinal plants have been used to enhance cognitive function and to alleviate other symptoms associated with AD. Plant constituents may not only act synergistically with other constituents from the same plant, but may also enhance the activity of compounds, or counteract toxic effects of compounds, from other plant species. Several milestones in the history of drug therapy have been discovered from ethnomedical knowledge, such as atropine, pilocarpine, cardiac glycosides, curare, and reserpine (Patel et al., 2014). In view of exploring the different sources for ethno-medical information, studies have resulted in discovery of new drugs for the treatment of AD and cognitive disorders among which, *C. asiatica* is one of the most important plants used in cognitive dysfunction (Brinkhaus et al., 2000).

Keeping the medicinal value of *C. asiatica* into consideration, present investigation was designed to perform a comparative study on organically and non-organically cultivated *C. asiatica*. The whole study was divided into two different segments i.e. phytochemical and pharmacological evaluations, where phytochemical evaluation was performed to obtain a comparative data on production of secondary metabolites of organically and non-organically cultivated *C. asiatica* at different time intervals of harvesting, while pharmacological evaluation will be helpful in selection of best optimized batch having potent memory enhancing activity. Therefore, the present study will be helpful in selecting the best fertilizers and pesticides and also best time of harvesting of the plant to produce the best quality *C. asiatica* with relatively higher potency.

2. Materials and methods

2.1. Cultivation and preparation

The cultivation of *C. asiatica* was carried out at the farm of Manas Ayurveda, Nagpur, situated about 46 km from Nagpur, Maharashtra, in the centre of the Indian peninsula 79° 7′ East longitude and 21° 7′ North latitude and is at a mean altitude of 310.5 m above sea level. The average annual rainfall of the area is 1205 mm. The average maximum temperature of the city is 33.53 °C and the average minimum temperature is 20.37 °C with relative humidity ranging between 20 and 70% (Dikshit, 1986). The cultivation of the plant was done through sowing the seeds procured from the local herbal market of Nagpur, Maharashtra, India.

2.1.1. Soil analysis

The soil of the experimental site was comprised of black clay soil with a pH of 6.8 and was analyzed for various mineral contents at Soil Testing Department of District Soil Survey and Soil Testing Laboratory, Nagpur, Maharashtra, India.

2.1.2. Preparation of manures

Organic manure was prepared using a mixture of protein rich materials including animal manures, fresh green grass, leaves and shoots of leguminous trees (*Cassia* species) and carbon rich materials including wood chips, dry leaves and grasses in a proportion of 60% protein and 40% carbonaceous matter. The heap was made in a layering method of about 2 m wide at the base, 0.5 m high and 3 m long, comprised of first stiff/hard layer of woody stems as a base. Later, it was followed by the carbonaceous layer of about 10 cm deep and a loose proteinaceous layer, which was about 15 cm deep. The animal dung was made into slurry and watered onto the carbon layer. Further, small amount of hydrated lime and rock powder was sprinkled over the pile. Heap was turned regularly for about 6 weeks and brown, crumbly humus was ready in 3 months (Itankar et al., 2016). Non-organic fertilizer viz. urea granules (N), super phosphate (P_2O_5) and potash (K_2O) per hectare was applied according to the treatment schedule as described by the manufacturer. (BHOODHAN - Granulated Mixture Fertilizers', marketed by Deepak Fertilizers' and Petrochemicals Corporation Limited (DFPCL), Maharashtra, India.

2.1.3. Preparation of pesticides

Fresh extract of the plants (*Adhatoda vasaka*, *Anamirta cocculus*, *Vitex negundo*, *Swertia chirata*, *Withania somnifera*, *Gardenia resinifera*, *Achyranthes aspera* and *Alstonia scholaris*), which are generally known for their fungicidal and insecticidal properties were used as an organic insecticide and fungicide. Soxhlet extraction method was used for plants, where ethanol was used as the solvent. Plant material except root, i.e. leaves, flowers, branches and stem was weighed and packed in filter paper as a pouch and kept in the Soxhlet. After completion of extraction, the extracts were obtained by removing ethanol through evaporation. The extracts were filtered and the filtrates were concentrated to dryness under reduced pressure with a vacuum rotary evaporator (IKA Germany) at 40 °C and stored under refrigeration until further use. Before use, each extract was re-suspended in their respective extracting solvents to yield 10 mL extract and was mixed into 1000 mL water, which was used as a natural pesticide for spraying. (Grange and Ahmed, 1988; Knight et al., 1997).

Monocrotophos 36% (Monocil, Insecticide India Ltd. India) and Zineb 75% W.P. (Indofil Z-78, Indofil Industries Ltd., Mumbai, India) were taken as non-organic insecticide and fungicide, which were purchased from local market and utilized in a ratio of 3:2 in water respectively (Anonymous, 2012).

2.2. Plant material and authentication

Leaves of the plant *C. asiatica* were collected on the basis of different time intervals of harvesting mainly after 1st, 2nd and 3rd month of cultivation. Authentication of the leaves was done by Dr. Nitin Dongarwar, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (Specimen number-9946). The collected leaves of the plant (500 g) were shade dried, coarsely powdered and successively extracted with ethanol (1.5 L) using Soxhlet extractor. The extraction was continued until the whole plant material was completely exhausted and the obtained extract was then concentrated and evaporated under reduced pressure in a rotary evaporator (IKA Germany).The extracts of *C. asiatica* collected at different time intervals of harvesting i.e. CCA: Control *C. asiatica* (collected from wild habitat), OCA1: Organic *C. asiatica* 1st harvesting, NOCA1: Non-organic *C. asiatica* 1st harvesting, OCA2: Organic *C. asiatica* 2nd harvesting, NOCA2: Non-organic *C. asiatica* 2nd harvesting, OCA3: Organic *C. asiatica* 3rd harvesting and NOCA3: Non-organic *C. asiatica* 3rd harvesting having % yield 5.73%, 6.90%, 6.23%, 6.33%, 5.72%, 6.42% and 5.93% w/w respectively, were then kept in a desiccator until use.

2.3. Macroscopic and microscopic evaluation

The macroscopical evaluation of the leaves was done by observing them with reference to their color, shape, size, odor and taste etc. (Fig. 1). For microscopical examination, the leaves of the plant were cut and were fixed in FAA (Formalin 5 mL + Acetic acid 5 mL + 70% Ethyl alcohol 90 mL) for about 24 h. Further, as per the schedule given by Sass (1940), the specimens were dehydrated with tertiary-butyl alcohol (TBA) followed by infiltration of the specimens by gradual addition of paraffin wax, until TBA solution attained super saturation. The specimens were then casted into paraffin blocks which were further sectioned with the help of Rotary Microtome at a thickness of 10–12 μm followed by dewaxing as described by Johansen (1940). Staining of the sections was done using Toludine blue (O'Brien et al., 1964).

2.4. Physiochemical standardization

The leaves of the plants were dried, ground and various physiochemical parameters of plant leaves were evaluated which includes, foreign matter, total ash, acid insoluble ash, water soluble ash, foaming index, extractive value, loss on drying, swelling index, pesticide content (Anonymous, 2002) and crude fiber content (Khandelwal, 2007).

Fig. 1. Leaves of *Centella asiatica.*

2.5. Phytochemical standardization

Preliminary phytochemical screening of the extracts of organically and non-organically cultivated *C. asiatica* extracts collected at different time intervals of harvesting were performed as per standard procedure (Trease and Evans, 2002). Further, total phenolic and tannin content in extract was estimated by using Folin ciocalteu reagent (Hagerman et al., 2000). The total flavonoid and flavanone contents were estimated following the methods of Kumaran and Karunakaran (2006), whereas total saponin content was estimated taking diosgenin as standard (Baccou et al., 1977). Total alkaloid content was estimated by gravimetric method (Wagner and Bladt, 1996), while total carbohydrates were estimated by adopting the method of Yemm and Willis (1954).

Ethanolic extracts of *C. asiatica* collected at different time intervals of harvesting i.e. CCA, OCA1, NOCA1, OCA2, NOCA2, OCA3 and NOCA3 were standardized with Asiatic acid (Sigma-Aldrich, St. Louis, MO, USA) using High Performance Thin Layer Chromatography (HPTLC). A stock solution of extracts (5 mg/mL) and Asiatic acid (0.5 mg/mL) were prepared in methanol. The mobile phase for developing the chromatogram consisted of Toluene: Ethyl acetate: Chloroform: Formic acid [5:4:1:0.5 v/v/v/v]. The study was carried out using Camag-HPTLC instrumentation (Camag, Mutten, Switzerland) equipped with Linomat V sample applicator, Camag TLC scanner 3, Camag TLC visualizer and WINCATS 4 software for data interpretation. The R_f values were recorded and the developed plate was screened and photo-documented at visible range after derivatization with methanolic sulphuric acid.

2.6. Experimental animals

Mice of the Swiss Albino strain (18 to 30 g in weight) of either sex were obtained from the Central Animal House (Reg. No. IAEC/UDPS/2014/21) of Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, India. The animals were kept in the experimental room for one week prior to testing and had free access to food and water ad libitum. Animals were housed 3–4 per cage in acclimatized conditions (25 ± 1 °C) and maintained on a 12/12 h light/dark cycle with lights on at 6:00 am. The behavioral tests were performed in the same room and all experiments were conducted during the daylight period. All experimental protocols were performed after approval from Central Animal Ethical Committee of Rashtrasant Tukadoji Maharaj Nagpur University (Letter No.: UDPS/2014/IAEC/21 dated 21/12/2014) and were conducted in accordance with accepted standard guidelines of National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

2.7. Acute oral toxicity study

The guideline proposed by Organization for Economic Co-operation and Development (OECD) 425 was used to determine the oral acute toxicity study. To the Swiss albino mice fasted for overnight, extracts were administered orally, as per the guidelines. The mice were then individually kept under observation for next 48 h with proper monitoring of any behavioral and neurological changes such as diarrhea, sleep, lacrimation, tremors, convulsions, salivation, drowsiness, sedation and feeding behavior as a sign of acute toxicity. To ascertain any rate of mortality, the mice were kept under observation up to 14 days (OECD, 2008).

2.8. Nootropic activity

2.8.1. Morris Water-Maze test

In case of Morris Water-Maze (MWM) test, animals were randomly divided into nine groups (n = 6). To the control group, vehicle [0.5 mL saline solution] was administered orally; to the positive control group Donipazil at a dose of 0.1 mg/kg p.o. was administered. To the rest of the groups, i.e. CCA, OCA1, NOCA1, OCA2, NOCA2, OCA3 and NOCA3, respective extracts were administered at dose levels of 100 mg/kg p.o. suspended in 0.4% CMC. The animals receiving the vehicle, extracts and standard drug were pre-treated for 4 days. The spatial reference memory (SRM) version of the MWM test was started on day 8, and consisted of 3 trial days and probe trial on day 11. After 2 days of resting period, the animals were subjected to spatial working memory (SWM) version of the MWM test. It consisted of acquisition and retrieval trials for 4 days i.e. day 14, 15, 16 and 17. Treatment was continued till the end of the experiment (i.e. day 17). Donipazil was administered 30 min before the SRM tests and continued until the end of SWM test (Morris, 1984; Davoodi et al., 2009).

2.8.2. Evaluation of nootropic activity using Morris Water-Maze test

The apparatus MWM is a fabricated white circular pool (120 cm in diameter, 45 cm high) filled with water to a depth of approximately 25 cm. The pool was divided into four equal quadrants and a platform (15 cm in diameter), which was submerged 0.5 cm below the opaque surface at the centre of one of the quadrants. The pool was located in a test room and many clues external to the maze were visible from the pool (e.g., pictures, lamps), which could be used by the mice for spatial orientation. The positions of the cues were kept constant throughout the task (Morris, 1984; Davoodi et al., 2009). Twenty-four hours prior to the start of training, all the mice were habituated to the pool by allowing them to perform a 60 s swim without the platform. SRM was tested after 7 days of pre-treatment; on day 8, 1 h after drug treatment, the mice were placed by hand into the water facing the wall of the pool, and were allowed 60 s to find the hidden platform. When successful, the mice were given 20 s on the platform to watch the spatial cues. Animals had 8 trials per day (separated by 10 min intervals), for 3 consecutive days. In each trial, the animal was released from a different start point in the pool, but the escape platform was kept on the same position (at the centre of the 4th quadrant). The time taken by the animals to reach the platform in each trial was recorded as escape latency. All recordings were done live by a blind observer. The probe trial (24 h later to last day of SRM) consisted of a 60 s free swim period without a platform in which the time spent in the target quadrant was recorded. Two days after the reference memory pre-training phase (i.e. on day 13), training on the working memory version of the navigation task was started. Only two trials per day were given over 4 consecutive days (days 13–16). In the first trial (acquisition), the animal was made to find the platform in a new position. In the second trial (retrieval), which was performed 75 min later, the platform was in its previous position but the animals were placed differently from that of the preceding trial (Davoodi et al., 2009). The time taken by the animal to reach the platform in both trials was recorded.

2.8.3. Estimation of AChE activity

After the assessment of nootropic activity by Morris Water-Maze test, all the animals were decapitated and the brains were removed immediately. Further, the discrete brain regions i.e. prefrontal cortex [PFC], hippocampus [HIP] and amygdale [AMY]) were dissected (Glowinski and Iversen, 1966) and homogenized. The total acetyl cholinesterase activity in the aliquot of the homogenate was estimated after mixing it with phosphate buffer (pH 7.0). To this, the substrate acetyl thiocholine iodide (Sigma, St. Louis, MO, USA) and dithiobisnitrobenzoic acid (DTNB) reagent (Sigma, St. Louis, MO, USA) were added. Acetylthiocholine iodide was hydrolyzed to thiocholine and acetate by AChE. Thiocholine reacted with DTNB reagent to produce a yellow color. The rate of color development is the measure of the AChE activity and was measured using UV–visible spectrophotometer at 412 nm at the interval of 15 s for 5 min. The enzyme activity is expressed as the 'n' moles of substrate hydrolyzed/min/mg of protein. The protein contents were determined in the brain samples as described by Lowry et al. (1951).

2.9. Statistical analysis

The experimental results are expressed as mean ± SEM, with six animals in each group followed by two-way analysis of variance (ANOVA). Bonferroni Post hoc test was applied for determining the statistical significance between different groups. Graph Pad Prism, version 5 software, was used for all statistical analysis. p values < 0.05 were considered to be significant.

3. Result

3.1. Soil analysis

In the present study, soil showed the neutral pH which may attributes to better fertility of plant. The soil also showed the presence of various minerals viz. salt, phosphorus, zinc, iron, potassium etc., which were detected in permissible limit, thus, justifying the better suitability of the soil for cultivation for *C. asiatica*.

3.2. Macroscopic and microscopic evaluation

The leaves of the plant *Centella asiatica* in macroscopical examination appeared to be rounded to reniform, 2 to 5 cm wide, horizontal, rounded at the tip, and kidney-shaped or heart shaped at the base, the rounded lobes often remain overlapped (Fig. 1). Transverse section of the leaves shows the dorsiventral nature of the leaf. A thin cuticle covers the upper epidermal cell. Mostly paracytic stomata are seen in surface view of both surfaces. No trichomes of any type whatsoever were observed. Palisade differentiated into two layers; spongy parenchyma consists of 3 layers with intercellular spaces and some cells may contain crystals of calcium oxalate. Midrib region shows 2 to 3 layers of collenchymas below the upper epidermis and above the lower epidermis. The vascular bundle is in centre and has xylem on the ventral side and phloem on the dorsal side. From the overall observation, the transverse section of all the samples of leaves collected at different time intervals of harvesting did not show any major differences.

3.3. Physicochemical properties

The parameters evaluated under physicochemical analysis (pH, foreign matter, loss on drying, swelling index, bitterness value, total ash, water soluble ash, acid insoluble ash, crude fiber and foaming index) of CCA, OCA1, NOCA1, OCA2, NOCA2, OCA3 and NOCA3 are represented in Table 1. The leaves of *C. asiatica* did show the presence of chlorinated and phosphated pesticides, which were found to be in the permissible range of 0.120 to 0.174 mg/kg for chlorinated and 0.013 to 0.022 mg/kg for phosphated pesticides in case of organic samples, while 0.219 to 0.410 mg/kg for chlorinated and 0.025 to 0.051 mg/kg for phosphate pesticide in case of nonorganic samples. The results clearly indicated higher quantities of pesticides in non-organic cultivated *Centella asiatica* compared to organic cultivated *Centella asiatica*, which justifies organic cultivation to be beneficial for human health care.

3.4. Phytochemical standardization

Preliminary phytochemical analysis of all the samples revealed the presence of phenols, flavonoids, flavonones, tannins, alkaloids, steroids and carbohydrates as major components. The quantitative estimations

Table 1
Physicochemical parameters of organically and non-organically cultivated *Centella asiatica* collected at different time intervals of harvesting.

Parameters	CCA	OCA1	NOCA1	OCA2	NOCA2	OCA3	NOCA3
Foreign –matter (% w/w)	4.95 ± 0.15	2.52 ± 0.11*	2.91 ± 0.43*	2.82 ± 0.65*	3.23 ± 0.18*	3.15 ± 0.16*	4.28 ± 23
Loss on drying (% w/w)	8.89 ± 0.21	9.82 ± 0.15	10.34 ± 0.31	8.45 ± 0.30	9.58 ± 0.44	7.23 ± 0.18*	8.11 ± 0.27
Swelling index (mL/g)	3.05 ± 0.26	2.41 ± 0.40*	2.83 ± 0.19	3.25 ± 0.22	3.82 ± 0.31	2.94 ± 0.16	3.78 ± 0.21
Bitterness value	Strong bitter	Strong bitter	Strong bitter	Strong bitter	Strong bitter	Strong bitter	Strong bitter
Total ash (% w/w)	21.86 ± 0.05	20.43 ± 0.10	20.91 ± 0.09	21.67 ± 0.18	22.13 ± 0.42	22.86 ± 0.16	23.57 ± 0.43
Water soluble ash (% w/w)	20.17 ± 0.31	15.40 ± 0.54	18.31 ± 0.17	18.97 ± 0.16	19.54 ± 0.29	20.28 ± 0.19	20.81 ± 0.32
Acid insoluble ash (% w/w)	3.36 ± 0.07	2.11 ± 0.12*	3.17 ± 0.15	2.80 ± 0.19*	3.54 ± 0.21	4.52 ± 0.18	4.85 ± 0.23
Crude fiber content (% w/w)	3.42 ± 0.11	1.77 ± 0.33*	2.22 ± 0.29	2.27 ± 0.22	3.25 ± 0.43	3.74 ± 0.23	4.37 ± 0.12
Foaming index	Less than 100	Less than 100	Less than 100	Less than 100	Less than 100	Less than 100	Less than 100

The values are represented as mean of triplicate readings ± S.E.M. (n = 3). In table * corresponds to $p < 0.05$ vs CCA. In table CCA: Control *C. asiatica* (collected from wild habitat), OCA1: Organic *C. asiatica* 1st harvesting, NOCA1: Non-organic *C. asiatica* 1st harvesting, OCA2: Organic *C. asiatica* 2nd harvesting, NOCA2: Non-organic *C. asiatica* 2nd harvesting, OCA3: Organic *C. asiatica* 3rd harvesting and NOCA3: Non-organic *C. asiatica* 3rd harvesting.

of all the identified phytoconstituents are represented in Table 2, where maximum quantity of secondary metabolites was observed in OCA1 and NOCA1. The results obtained from the HPTLC quantification revealed the presence of Asiatic acid in ethanolic extracts of all the samples. The quantity of Asiatic acid in all the samples was determined to be as CCA: 4.56, OCA1: 8.50, NOCA1: 7.67, OCA2: 6.46, NOCA2: 5.31, OCA3: 3.09, NOCA3: 1.42% w/w respectively. Thus, from the overall observation, organically cultivated *C. asiatica* collected after 1st month of harvesting showed the maximum amount of Asiatic acid compared to other samples (Fig. 2).

3.5. Acute oral toxicity study

The acute oral toxicity study proved the extract to be safe up to 2 g/kg showing no signs of toxicity as depicted through observations of behavioral pattern, which were found to be normal in treated mice.

3.6. Nootropic activity

On the basis of results from acute oral toxicity study and preliminary screening for nootropic activity, ethanolic extract of all the batches of *C. asiatica* at 100 mg/kg p.o. was selected for performing the final nootropic activity. The nootropic activity primarily included determination of Escape latency, where the results depicted a significant ($p < 0.05$) reduction in escape latency with respect to time among groups treated with OCA1 and NOCA1, which showed (Table 3) more potent activity, quite similar to that of standard drug Donipazil (0.1 mg/kg p.o.). Further the study included determination of probe trial, which was conducted 24 h after training period, wherein the time spent by the animal in the target quadrant was determined. From the statistical analysis, the result demonstrated a significant increase among the treated group as compared to control animal in terms of time spent in the target quadrant. It may be attributed to an enhanced spatial memory that may have helped treated animals in locating during previously placed platform in quadrant, compared to animal in control group.

In determination of spatial working memory, which was conducted on day 14 of our treatment schedule, two different versions of memories i.e. acquisition and retrieval trial were conducted. From the statistical analysis, the result demonstrated a significant reduction in escape latency of treated group, which was reported highest in case of OCA1, NOCA1 and Donipazil with reference to acquisition as well as retrieval version of memory, indicating potential nootropic activity of OCA1 and NOCA1 (Table 4). From the overall results, maximum effect was observed in group treated with standard Donipazil (0.1 mg/kg p.o).

Fig. 3 illustrated the effect of OCA1 and NOCA1 (100 mg/kg p.o) on the AChE activity in different brain regions namely PFC, HIP and AMY. Post hoc analysis has shown that OCA1 and NOCA1 treated groups significantly inhibited the AChE activity compared to that of control in all the brain regions tested, which was more prominent in case of OCA1 treatment.

4. Discussion

India has varied agro-climatic conditions, which make it suitable for growing a wide range and variety of valuable medicinal plants. However, the higher cost of production as compared to the material collected illegally from the forests, unstable demand, slow growth rates and low prices paid for traditional medicines hardly make cultivation of medicinal plants a profitable exercise. These factors are luring the farmers towards use of chemical fertilizers to increase the yield in a short span of time (Cunningham, 1993). Though, chemical fertilizers increase the yield, they pose certain serious health threats to human beings especially infants, pregnant and nursing mothers (Vermeer et al., 1998). To avoid such problems, recently there is shift of people towards organic farming, which implies use of pesticides and fertilizers from natural source. The practice has resulted in elimination of health problems associated with inorganic cultivation. In contrast, organic manures are considered to be safe and ecofriendly by improving water penetration, water holding capacity, improvement in soil structure, microbial biomass, nutrient availability, drought and heat stress resistance. It also helps in improving the soil pH, which has an impact on plant growth and soil microbial activity (Cantisano, 2000). Keeping above view into consideration, the present investigation was designed to perform a comparative evaluation of organic and non-organic cultivated *C. asiatica* collected at different time intervals of harvesting with reference to phytochemical and preclinical profiling. *C. asiatica* is one of the plants having very high

Table 2
Quantitative estimations of phytochemicals in organically and non-organically cultivated *Centella asiatica* collected at different time intervals of harvesting.

Phytochemicals estimated	CCA	OCA1	NOCA1	OCA2	NOCA2	OCA3	NOCA3
Total phenolic content (mg/g gallic acid equivalent)	109.32 ± 0.23	145.25 ± 0.27*	122.77 ± 0.16*	112.81 ± 0.23	111.91 ± 0.09	105.82 ± 0.12	104.46 ± 0.31
Total alkaloid content (% w/w in plant material)	0.29 ± 0.18	1.11 ± 0.23*	0.18 ± 0.21	1.01 ± 0.13*	0.19 ± 0.24	0.26 ± 0.15	0.38 ± 0.06
Total flavonoid content (mg/g rutin equivalent)	120.73 ± 0.17	301.12 ± 0.05*	287.12 ± 0.02*	170.43 ± 0.06*	140.65 ± 0.05	130.87 ± 0.11	129.24 ± 0.07
Total flavanone content (mg/g naringine equivalent)	35.41 ± 0.10	95.65 ± 0.12*	70.12 ± 0.31*	50.98 ± 0.09	45.90 ± 0.14	33.64 ± 0.10	37.19 ± 0.07
Total carbohydrates (mg/g d fructose equivalent)-	7.80 ± 0.12	27.54 ± 0.08*	16.03 ± 0.03*	22.17 ± 0.08*	21.28 ± 0.12*	12.51 ± 0.21	8.47 ± 0.06
Total tannin content (mg/g tannic acid equivalent)	1.11 ± 0.02	5.82 ± 0.23*	3.03 ± 0.11*	4.10 ± 0.03*	2.01 ± 0.14	1.42 ± 0.31	1.21 ± 0.24

The values are represented as mean of triplicate readings ± S.E.M. (n = 3). In table * corresponds to $p < 0.05$ vs CCA. In table CCA: Control *C. asiatica* (collected from wild habitat), OCA1: Organic *C. asiatica* 1st harvesting, NOCA1: Non-organic *C. asiatica* 1st harvesting, OCA2: Organic *C. asiatica* 2nd harvesting, NOCA2: Non-organic *C. asiatica* 2nd harvesting, OCA3: Organic *C. asiatica* 3rd harvesting and NOCA3: Non-organic *C. asiatica* 3rd harvesting.

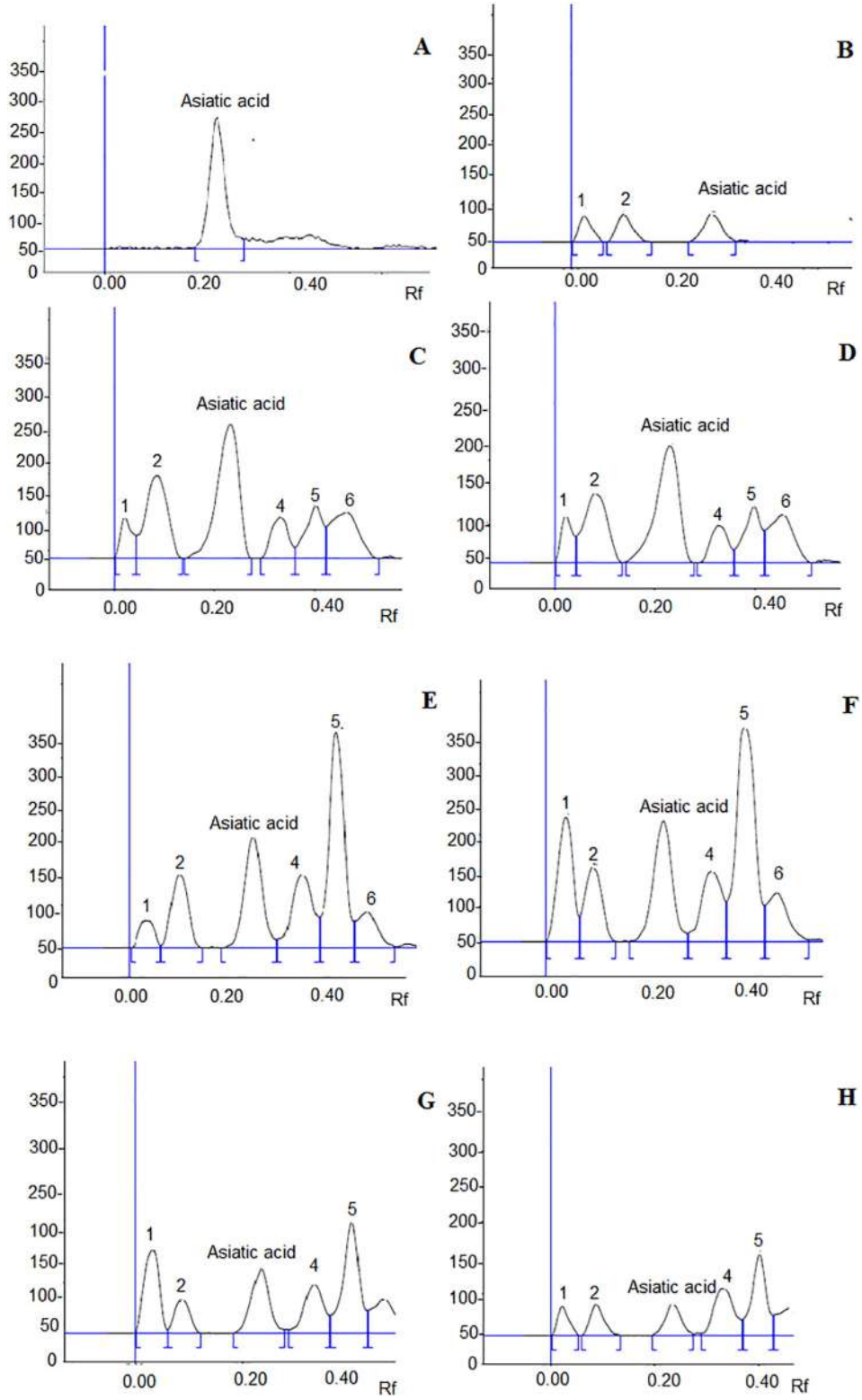

Fig. 2. HPTLC chromatogram of organically and non-organically cultivated *Centella asiatica* collected at different time intervals of harvesting showing the presence of Asiatic acid. In panel A: Standard peak of Asiatic acid, B: Control *C. asiatica* (collected from wild habitat (CCA)), C: Organic *C. asiatica* 1st harvesting (OCA1), D: Non-organic *C. asiatica* 1st harvesting (NOCA1), E: Organic *C. asiatica* 2nd harvesting (OCA2), F: Non-organic *C. asiatica* 2nd harvesting (NOCA2), G: Organic *C. asiatica* 3rd harvesting (OCA3) and H: Non-organic *C. asiatica* 3rd harvesting (NOCA3).

medicinal value such as memory enhancing, anti-amnestic, antioxidant, antidiabetic, antihypertensive, anticancer properties (Yasurin et al., 2016). Even though, the plant has a very high medicinal value, there is no data available on its impact on active phytoconstituents with respect to its organic and inorganic cultivation along with different time intervals of harvesting.

Soil is an important parameter for the cultivation of medicinal plant, which provides required nutrients for producing medicinally rich plants

Table 3
Effect of organically and non-organically cultivated *Centella asiatica* collected at different time intervals of harvesting on escape latencies on training days 1, 2 and 3 in SRM Version of MWM test from starting quadrants (SQ) to NE, NW, SE and SW quadrants.

Groups	Starting quadrants					
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	North East (L-1) (NE)			North West (R-1) (NW)		
Control	60.08 ± 2.20	45.83 ± 1.94	35.86 ± 1.22	40.32 ± 3.34	31.23 ± 1.23	22.14 ± 1.09
Donipazil	44.56 ± 1.55*	30.55 ± 1.22*	15.55 ± 1.14*	30.34 ± 2.21*	22.34 ± 1.32*	10.21 ± 1.67*
CCA	51.32 ± 2.89*	37.01 ± 1.60*	20.23 ± 1.44*	34.98 ± 1.46*	24.54 ± 1.90*	17.38 ± 1.67*
OCA1	49.89 ± 1.43*	35.43 ± 1.24*	17.34 ± 1.09*	33.21 ± 1.21*	23.43 ± 2.31*	13.32 ± 1.42*
NOCA1	51.08 ± 1.47*	37.98 ± 1.29*	19.33 ± 1.15*	34.16 ± 1.76*	22.21 ± 1.45*	14.98 ± 1.22*
OCA2	51.72 ± 1.31*	37.47 ± 1.21*	20.32 ± 1.13*	36.45 ± 2.33*	23.45 ± 1.23*	17.34 ± 0.34*
NOCA2	54.23 ± 1.42*	38.78 ± 1.32*	21.32 ± 1.19*	35.94 ± 1.69*	24.03 ± 1.89*	18.23 ± 1.78*
OCA3	56.70 ± 1.66*	39.36 ± 1.28*	21.31 ± 1.18*	37.42 ± 1.89*	26.32 ± 2.33*	19.22 ± 1.90*
NOCA3	57.32 ± 1.89*	40.11 ± 1.67*	22.23 ± 1.34*	37.98 ± 1.45*	27.54 ± 1.95*	19.38 ± 1.67*
	South East (L-2) (SE)			South West (R-2) (SW)		
Control	59.08 ± 1.90	41.83 ± 1.94	30.06 ± 2.02	39.12 ± 1.34	27.03 ± 1.23	20.14 ± 1.19
Donipazil	44.88 ± 1.30*	28.55 ± 1.22*	9.55 ± 1.04*	29.14 ± 1.11*	20.34 ± 1.32*	8.21 ± 0.57*
CCA	50.88 ± 2.39*	33.11 ± 1.77*	17.13 ± 1.20*	35.08 ± 1.55*	24.14 ± 1.02*	13.98 ± 0.60*
OCA1	47.19 ± 2.33*	30.43 ± 1.14*	11.63 ± 0.79*	33.11 ± 0.71*	20.21 ± 1.31*	9.02 ± 0.82*
NOCA1	50.88 ± 1.47*	32.18 ± 2.19*	12.93 ± 0.95*	34.06 ± 0.76*	22.41 ± 1.25*	11.08 ± 1.42*
OCA2	50.72 ± 2.34*	34.47 ± 2.21*	15.31 ± 1.01*	34.15 ± 1.76*	23.45 ± 1.23*	11.94 ± 0.74*
NOCA2	51.23 ± 1.42*	36.78 ± 1.32*	15.02 ± 0.92*	34.04 ± 1.39*	24.03 ± 1.89*	11.23 ± 0.98*
OCA3	50.70 ± 1.76*	33.36 ± 1.18*	17.91 ± 1.81*	35.12 ± 0.89*	22.12 ± 1.26*	11.22 ± 1.30*
NOCA3	51.88 ± 1.89*	32.11 ± 1.67*	18.13 ± 1.24*	37.08 ± 1.05*	24.14 ± 1.02*	12.98 ± 0.67*

Each value represents mean ± SEM (n = 6), *p < 0.05 compared to control (two-way ANOVA followed by Bonferroni post hoc test). In table CCA: Control *C. asiatica* (collected from wild habitat), OCA1: Organic *C. asiatica* 1st harvesting, NOCA1: Non-organic *C. asiatica* 1st harvesting, OCA2: Organic *C. asiatica* 2nd harvesting, NOCA2: Non-organic *C. asiatica* 2nd harvesting, OCA3: Organic *C. asiatica* 3rd harvesting and NOCA3: Non-organic *C. asiatica* 3rd harvesting.

products. Soil contents have been reported to have significant role in terms of its observed pharmacological response (Palm et al., 2001). In the present study, soil showed a neutral pH which attributes to better fertility of plant. The other parameters like salt, phosphorus, zinc, iron potassium etc. were detected in permissible limit, thus supporting a healthy environment for cultivation for *C. asiatica*.

The first primary objective of our present study was to develop a standard quality control profile of *C. asiatica*. Macroscopical examination of a plant/plant parts represents detailed information regarding the qualitative assessment of plant based on its morphological and sensory characters such as size, shape color, taste, odor while microscopical evaluation provides us extensive knowledge about the cellular arrangement of tissues (Neogi et al., 1989). Microscopy of the leaf revealed that the leaf is dorsiventral, with palisade cells below the upper epidermis. Microscopic study could not reveal any noticeable changes between organic and non-organic cultivated *C. asiatica*. Total ash in a plant material includes both physiological and non-physiological ash, while acid insoluble ash is a part of total ash and is an indicative of silica present, especially as sand and siliceous earth whereas, water soluble ash is the water soluble portion of the total ash (Prasad et al., 2013). The plant showed the presence of higher quantity of total ash in samples obtained after third harvesting of organic and non-organic *C. asiatica*, which is an indication of mixture of physiological and non-physiological ash. It is very essential to control the moister content, since higher moisture content in plant material may lead to its deterioration and may therefore result in percentage variation of active constituents. Swelling index of a plant material is conclusive of the therapeutic or pharmaceutical value which may be attributed to the presence of gums, mucilage, pectin and hemicelluloses (Prasad et al., 2013). As far as proximate analysis is concerned, OCA1 and NOCA1 showed a moderate to low amount of crude fiber, foreign matter, loss on drying and swelling index.

Agricultural practice such as spraying and treatment of soils occurring throughout the processes of cultivation, and administration of fumigants during storage may result in contamination of medicinal plant/plant parts with pesticides. WHO has therefore, suggested that every nation dealing with production of medicinal plants/plant parts should have at least one central laboratory which will provide information regarding standard limits of pesticides (Anonymous, 2002). The leaves of *C. asiatica* did show the presence of chlorinated and phosphated pesticides however they were present in accordance with the standard limits of these pesticides. Phytochemical screening gives the idea about the phytoconstituents present in the plants. The therapeutic value of the plants is attributed to its active constituents, which are being investigated to serve as pharmacological tool to provide

Table 4
Effect of organically and non-organically cultivated *Centella asiatica* collected at different time intervals of harvesting on percentage time spent in target quadrant during probe trial.

Groups	Day 1		Day 2		Day 3		Day 4	
	Acquisition	Retrieval	Acquisition	Retrieval	Acquisition	Retrieval	Acquisition	Retrieval
Control	51.23 ± 2.33	40.34 ± 2.09	44.33 ± 1.97	39.72 ± 1.34	42.36 ± 1.87	35.23 ± 1.66	40.21 ± 1.56	29.43 ± 0.98
Donipazil	39.02 ± 1.98*	28.21 ± 0.76*	34.39 ± 1.07*	20.43 ± 0.54*	28.12 ± 1.21*	14.77 ± 0.32*	22.30 ± 1.08*	9.36 ± 0.12*
CCA	42.64 ± 2.55*	33.23 ± 1.10*	38.55 ± 1.88*	29.67 ± 1.83*	38.00 ± 2.01*	26.94 ± 1.75*	33.07 ± 1.54*	18.23 ± 0.97*
OCA1	40.32 ± 1.87*	30.99 ± 0.99*	35.34 ± 1.54*	23.99 ± 0.67*	29.99 ± 0.76*	16.34 ± 0.44*	22.02 ± 0.21*	10.45 ± 0.13*
NOCA1	42.74 ± 1.77*	32.09 ± 1.19*	36.23 ± 1.76*	24.56 ± 1.08*	30.98 ± 0.92*	18.06 ± 0.41*	24.46 ± 0.41*	12.45 ± 0.21*
OCA2	42.33 ± 1.97*	34.32 ± 1.78*	40.31 ± 1.88*	29.44 ± 1.10*	36.98 ± 1.21*	20.37 ± 1.20*	29.44 ± 0.85*	15.34 ± 0.18*
NOCA2	44.52 ± 1.32*	38.44 ± 1.29*	41.23 ± 2.01*	33.90 ± 1.32*	37.43 ± 1.13*	25.44 ± 0.92*	30.23 ± 0.98*	19.34 ± 0.34*
OCA3	45.21 ± 2.11*	39.42 ± 1.72	42.31 ± 2.01*	31.23 ± 1.13*	39.99 ± 1.91*	29.34 ± 1.09*	33.12 ± 1.23*	20.78 ± 0.41*
NOCA3	45.34 ± 2.51*	40.13 ± 2.10	42.45 ± 1.98	34.77 ± 1.43*	40.10 ± 2.21	30.94 ± 1.35*	34.97 ± 1.34*	21.23 ± 0.87*

Each value represents mean ± SEM (n = 6), *p < 0.05 compared to control (two-way ANOVA followed by Bonferroni post hoc test). In table CCA: Control *C. asiatica* (collected from wild habitat), OCA1: Organic *C. asiatica* 1st harvesting, NOCA1: Non-organic *C. asiatica* 1st harvesting, OCA2: Organic *C. asiatica* 2nd harvesting, NOCA2: Non-organic *C. asiatica* 2nd harvesting, OCA3: Organic *C. asiatica* 3rd harvesting and NOCA3: Non-organic *C. asiatica* 3rd harvesting.

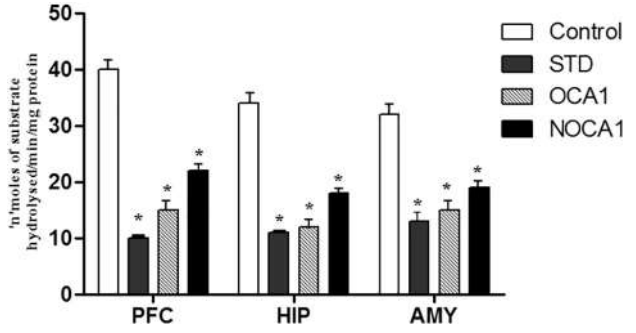

Fig. 3. Effect of organically (OCA1) and non-organically (NOCA1) cultivated *Centella asiatica* collected after first month of harvesting on acetylcholinesterase activity in different brain regions i.e. prefrontal cortex (PFC), hippocampus (HIP) and amygdale (AMY). Values are expressed in mean ± SEM (n = 6), where * corresponds to $p < 0.05$ vs Control. In figure STD corresponds to standard drug used Donipazil at 0.1 mg/kg.

health and wellness (Liu, 2005). In this connection, preliminary phytochemical screening of ethanolic extracts of all the samples from *C. asiatica* were performed which showed most prominent presence of flavonoids, glycosides, polyphenols and carbohydrate. The quantitative estimations of phytochemicals suggested the extracts to be highly rich in total phenolic and flavonoids, while tannins were present in considerable amount. From the overall observation, the samples collected from the first harvesting showed maximum quantity of phytoconstituents. In addition, lower quantities of secondary metabolites as observed in non-organic crop may be due to the interference in normal biosynthetic pathways by the higher amount of heavy metals (Singh et al., 2011). The results obtained from the HPTLC quantification showed that, Asiatic acid was present in ethanolic extracts of all samples. However, the observation demonstrated that, organically cultivated leaves of *C. asiatica* collected after 1st harvesting showed the maximum amount of Asiatic acid. Asiatic acid is a triterpenoid reported to have memory enhancing activity, antioxidant, wound healing properties (Nasir et al., 2011). Due to these properties of Asiatic acid, it was used as a chemical marker for the standardization of different extracts obtained from the leaves of C. *asiatica*.

For pharmacological evaluations, MWM test was performed to examine spatial learning and memory (Morris, 1984; D'Hooge and De Deyn, 2001) in rodents. In the MWM test, it is assumed that, decrease in the escape latency in training trial and longer time spent in the target quadrant during probe trial were linked with the enhancement in the spatial learning and memory (Morris, 1984). Spatial memory is a part of the memory responsible for recording information about one's environment and its spatial orientation and is formed after an organism gathers and processes sensory information about its surroundings. In the present investigation, 4 days pre-treatment with all the extracts and Donipazil (0.1 mg/kg) decreased escape latency on all 3 days of training trials. To ascertain the retention of this memory, further probe trial was conducted after 24 h of training trials without the hidden platform. OCA1, NOCA1, OCA2, NOCA2 and OCA3 treated groups significantly increased the time spent in the target quadrant during the probe trial, indicating retention of spatial memory of the location of previously placed platform in the target quadrant as compared to the control animals. However, compared to all the groups, OCA1 treated mice were found to have more prominent effect, suggesting a very high efficiency of OCA1 in facilitating reference memory and increasing learning capability of experimental animal. In both the SRM and the probe trial, 100 mg/kg., p.o. doses of OCA1, remarkably improved spatial and retention of memory and this effect was quite comparable with that of standard Donipazil.

Working memory is a theoretical construct within cognitive psychology, used for temporarily storing and manipulating information in short-term memory. In the SWM, version of memory testing, during the first trial i.e. acquisition trial, the animal had to locate the platform in a new place, whereas in the retrieval trial, which was carried out 75 min after acquisition trial, the platform was in its preceding position (target quadrant) (Sarihi et al., 2000). Treatment with ethanolic extract of all samples from *C. asiatica* appreciably decreased the escape latency time in acquisition and retrieval trials suggesting improvement in the SWM in the mice. The study also revealed that, OCA1 and NOCA1 depicted a more enhanced spatial reference as well as working memory in the MWM test. As OCA1 and NOCA1 improved memory in the absence of any cognitive deficit, it can be recommended to possess enhanced nootropic activity as compared to other *C. asiatica* samples.

Roland et al. (2008) demonstrated that cholinesterase inhibitors can enhance behavioral performance. In the above context, and on the basis of observed results, we evaluated the effect of the OCA1 and NOCA1 on AChE activity. In the present study, OCA1 and NOCA1 pre-treated groups significantly decreased AChE activity in all the brain regions namely HIP, PFC and AMY. The HIP has been shown to be related with memory processing such as declarative memory (Squire, 1992), spatial memory (O'Keefe and Nadel, 1978), recognition memory (Gaffan, 1974) and developing episodic-like memory (Morris, 2006). By influencing perception and attention, the AMY can alter the encoding of hippocampal dependent, episodic memory, so that emotional events receive priority (Davis and Whalen, 2001). PFC is critical component responsible for working memory and has been found that, encoding of long-term memories preferentially activates the left PFC, whereas retrieval of those memories activate the right PFC (Buckner, 1996). It has been reported that decrease in AChE activity indicates an increase in the basal level of ACh (Yamada et al., 2004). Thus, highly significant increase in ACh by OCA1 as compared to NOCA1 by virtue of its anti-AChE activity in the above brain regions confirms its better nootropic activity. In addition, Asiatic acid was also reported to be present in higher quantity in OCA1. Asiatic acid as a nootropic agent, acts as GABA-β agonist and a potent AChE inhibitor, that helps in controlling the level of Ach in the hippocampus which is important to memory and learning abilities (Nasir et al., 2011).

Thus, from the present investigation, we have justified the preference of organic over non-organic cultivation of *C. asiatica* and have also proved that, the leaves collected after first month of cultivation contains maximum amount of secondary metabolites as compared the other time interval of harvesting showing best memory enhancing potential.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the writing and content of the paper.

Acknowledgment

The authors would like to acknowledge the valuable support of Mr. Amrish Ghatate from Manas Ayurveda, Nagpur, Maharashtra, India for providing facilities for cultivation of *C. asiatica*.

References

Anonymous, 2002. Quality Control Methods for Medicinal Plant Materials (An Authorized Publication of World Health Organization, Geneva). A.I.T.B.S. Publishers & Distributors (Regd), Delhi.

Anonymous, 2012. Krushi Sanvardhini. 5th ed. Dr. Punjabrao Deshmukh Agricultural University, Akola, India.

Baccou, J.C., Lambert, F., Sauvaire, Y., 1977. Spectrophotometric method for the determination of total steroidal sapogenin. Analyst 102, 458–465.

Brinkhaus, B., Lindner, M., Schuppan, D., Hahn, E.G., 2000. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine 5, 427–448.

Buckner, R., 1996. Beyond HERA: contributions of specific prefrontal brain areas to long-term memory retrieval. Psychon. Bull. Rev. 3, 149–158.

Bunpo, P., Kataoka, K., Arimochi, H., Nakayama, H., Kuwahara, T., Bando, Y., Izumi, K., Vinitketkumnuen, U., Ohnishi, Y., 2004. Inhibitory effects of *Centella asiatica* on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rat. Food Chem. Toxicol. 42, 1987–1997.

Cantisano, A., 2000. Know Your Soil, a Handbook for Organic Growers and Gardeners in Temperate and Sub-tropical Regions. Organic Ag Advisors, Colfax, CA, pp. 1–2 (Edited and adopted by Julian Dumanski, RDV, World Bank).
Chandrika, U.G., Prasad Kumarab, P.A., 2015. Gotu kola (*Centella asiatica*): nutritional properties and plausible health benefits. Adv. Food Nutr. Res. 76, 125–157.
Cheng, C.L., Guo, J.S., Luk, J., Koo, M.W.L., 2004. The healing effects of *Centella* extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Sci. 74, 2237–2249.
Cunningham, A.B., 1993. An Africa-wide Overview of Medicinal Plant Harvesting, Conservation and Health Care. WWF/UNESCO/New People and Plants initiative, South Africa, pp. 116–118.
Davis, M., Whalen, P.J., 2001. The amygdala: vigilance and emotion. Mol. Psychiatry 6, 13–34.
Davoodi, F.G., Motamedi, F., Naghdi, N., Akbari, E., 2009. Effect of reversible inactivation of the reuniens nucleus on spatial learning and memory in rats using Morris water maze task. Behav. Brain Res. 198, 130–135.
D'Hooge, R., De Deyn, P.P., 2001. Applications of the Morris water maze in the study of learning and memory. Brain Res. Rev. 36, 60–90.
Dikshit, K.R., 1986. Maharashtra in Maps. Maharashtra State Board for Literature and Culture, Bombay, India.
Ekor, M., 2014. The growing use of herbal medicines: issues relating to adverse challenges in monitoring safety. Front. Pharmacol. 4, 1–10.
Firuzi, O., Pratic, D., 2006. Coxibs and Alzheimer's disease: should they stay or should they go? Ann. Neurol. 59, 219–228.
Gaffan, D., 1974. Recognition impaired and association intact in the memory of monkeys after transection of the fornix. J. Comp. Physiol. Psychol. 86, 1100–1109.
Giurgea, C., 1973. The nootropic approach to the pharmacology of the integrative action of the brain. Cond. Reflex 8, 108–115.
Glowinski, J., Iversen, L.L., 1966. Regional studies of catecholamines in the rat brain I. The disposition of [^{3}H]norepinephrine, [^{3}H] dopamine and [^{3}H]dopa in various regions of the brain. J. Neurochem. 13, 655–669.
Grange, M., Ahmed, S., 1988. Handbook of Plants with Pest Control Properties. John Wiley & Sons, New York.
Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., 2000. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. Food Chem. 46, 1887–1892.
Itankar, P., Tauqeer, M., Brahmankar, C., 2016. Cultivation, nutritional, antinutritional, and mineral analysis of organic and non-organic *Momordica charantia*: comparative study. Int. J. Green Pharm. 10, 136–144.
Johansen, D.A., 1940. Plant Microtechnique. Mc Graw Hill Book Co., New York, p. 523.
Juan, C., Molto, J.C., Lino, C.M., Manes, J., 2007. Determination of ochratoxin A in organic and non-organic cereals and cereal products from Spain and Portugal. Food Chem. 107, 525–530.
Khandelwal, K., 2007. Practical Pharmacognosy, Techniques and Experiments. 17th edition. Nirali Prakashan, Pune, India.
Knight, S.C., Anthony, V.M., Brady, A.M., Greenland, A.J., Heaney, S.P., Murray, D.C., Powell, K.A., Schulz, M.A., Spinks, C.A., Worthington, P.A., Youle, B., 1997. Rationale and perspectives on the development of fungicides. Annu. Rev. Phytopathol. 35, 349–372.
Kumaran, A., Karunakaran, J., 2006. In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India. LWT Food Sci. Technol. 40, 344–352.
Lee, Y.S., Jin, D.Q., Kwon, E.J., Park, S.H., Lee, E.S., Jeong, T.C., Nam, D.H., Huh, K., Kim, J.A., 2002. Asiatic acid, a triterpene, induces apoptosis through intracellular Ca^{2+} release and enhanced expression of p53 in HepG2 human hepatoma cells. Cancer Lett. 186, 83–91.
Liu, J., 2005. Oleanolic acid and Ursolic acid: Reserch perspectives. J. Ethnopharmacol. 100, 92–94.
Lohr, L., 2001. Factors affecting international demand and trade in organic food products. In: Regmi, A. (Ed.), Changing Structure of Global Food Consumption and Trade. USDA/Economic Research Service, Washington, DC, pp. 67–79.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60.
Morris, R.G., 2006. Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. Eur. J. Neurosci. 23, 2829–2846.
Nasir, M.N., Habsah, M., Zamzuri, I., Rammes, G., Hasnan, J., Abdullah, J., 2011. Effects of asiatic acid on passive and active avoidance task in male Spraque–Dawley rats. J. Ethnopharmacol. 134, 203–2091.
Neogi, B., Prasad, M.N.V., Rao, R.R., 1989. Ethnobotany of some weeds of Khasi and Garo hills, Meghalaya, Northeastern India. Econ. Bot. 43, 471–479.
O'Brien, T.P., Feder, N., McCully, M.E., 1964. Polychromatic staining of plant cell walls by Toluidine Blue-O. Protoplasma 59, 368–373.
OECD, 2008. Guidelines for testing of chemicals. Guidance no. 425. Acute oral toxicity: up and down procedure (UDP). http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECDtg425.pdf (S (adopted 03.10.08)).
O'Keefe, J., Nadel, L., 1978. The Hippocampus as a Cognitive Map. Oxford University Press, Oxford, United Kingdom.
Orhan, I.E., 2012. *Centella asiatica* (L.) Urban: from traditional medicine to modern medicine with neuroprotective potential. Evid. Based Complement. Alternat. Med. 1–8.
Orhan, I.E., Atasu, E., Senol, F.S., Ozturk, N., Demirci, B., Das, K., Sekeroglu, N., 2013. Comparative studies on Turkish and Indian *Centella asiatica* (L.) Urban (gotu kola) samples for their enzyme inhibitory and antioxidant effects and phytochemical characterization. Ind. Crop. Prod. 47, 316–322.
Palm, C.A., Gachengo, C.N., Delve, R.J., Cadisch, G., Giller, K.E., 2001. Organic inputs for soil fertility management in tropical agroecosystems: application of an organic resource database. Agric. Ecosyst. Environ. 83, 27–42.
Patel, V.S., Jivani, N.P., Patel, S.B., 2014. Medicinal plants with potential nootropic activity: a review. Res. J. Pharm., Biol. Chem. Sci. 5, 1–11.
Pingale, S.S., 2008. Evaluation of effect of *Centella asiatica* on CCL4 induced rat liver damage. Pharmacology 3, 537–543.
Prasad, S.K., Laloo, D., Kumar, M., Hemalatha, S., 2013. Quality control standardization and antioxidant activity of roots from *Eriosema chinense*. Pharmacogn. J. 5, 149–155.
Ranade, A.V., Acharya, R., 2015. Influence of time factor on phytoconstituents in certain ayurvedic medicinal plant: a comprehensive review. J. Pharm. Sci. Innov. 4, 235–341.
Roland, J.J., Mark, K., Vetreno, R.P., Savagea, L.M., 2008. Increasing hippocampal acetylcholine levels enhance behavioral performance in an animal model of diencephalic amnesia. Brain Res. 1234, 116–127.
Sampson, J.H., Raman, A., Karlsen, G., Navsaria, H., Leigh, I., 2001. In vitro keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins. Phytomedicine 8, 230–235.
Sarihi, A., Motamedi, F., Naghdi, N., Rashidy-Pour, A., 2000. Lidocaine reversible inactivation of the median raphe nucleus has no effect on reference memory but enhances working memory versions of the Morris water maze task. Behav. Brain Res. 114, 1–9.
Sass, J.E., 1940. Elements of Botanical Microtechnique. Mc Graw Hill Book Co., New York, p. 222.
Singh, A., Singh, R.D., Awasthi, R.P., 1996. Organic Andinorganic sources of fertilizers for sustained productivity in Rice (*Oryza sativa*)-wheat (*Triticum aestivum*) sequences on humid hilly soils of Sikkim. Indian J. Agron. 41, 191–194.
Singh, R., Gautam, N., Mishra, A., Gupta, R., 2011. Heavy metals and living systems: an overview. Indian J. Pharm. 43, 246–253.
Somchit, N.M., Sulaiman, R.M., Zuraini, A., Samsuddin, L., Somchit, N., Israf, A.D., Moin, S., 2004. Antinociceptive and antiinflammatory effects of *Centella asiatica*. Indian J. Pharm. 36, 377–380.
Soni, U., Brar, S., Gauttam, V., 2015. Effect of seasonal variation on secondary metabolites of medicinal plants. Int. J. Pharm. Sci. Res. 6, 3654–3662.
Squire, L.R., 1992. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol. Rev. 99, 195–231.
Sudha, S., Kumaresan, S., Amit, A., David, J., Venkataraman, V.B., 2002. Anti-convulsant activity of different extracts of *Centella asiatica* and *Bacopa monnieri* in animals. J. Nat. Rem. 2, 33–41.
Suguna, L., Sivakumar, P., Chandrakasan, G., 1996. Effects of *Centella asiatica* extract on dermal wound healing in rats. Indian J. Exp. Biol. 34, 1208–1211.
Trease, G.E., Evans, W.C., 2002. Pharmacognosy. 15th edition. W.B. Saunders Company Ltd., London.
Vermeer, I.T., Pachen, D.M., Dallinga, J.W., Kleinjans, J.C., Van Maanen, J.M., 1998. Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. Environ. Health Perspect. 106, 459–463.
Wagner, H., Bladt, S., 1996. Plant drug analysis. 2nd ed. Springer-Verlog Berlin Heidelberg, Germany.
Wang, X.S., Dong, Q., Zuo, J.P., Fang, J.N., 2003. Structure and potential immunological activity of pectin from *Centella asiatica* (L.) Urban. Carbohydr. Res. 338, 2393–2402.
Wijeweera, P., Arnason, T.J., Koszycki, D., Merali, Z., 2006. Evaluation of anxiolytic properties of Gotukola—(*Centella asiatica*) extracts and asiaticoside in rat behavioral models. Phytomedicine 13, 668–676.
Yamada, N., Hattori, A., Hayashi, T., Nishikawa, T., Fukuda, H., Fujino, T., 2004. Improvement of scopolamine-induced memory impairment by Z-ajoene in the water maze in mice. Pharmacol. Biochem. Behav. 78, 787–791.
Yasurin, P., Sriariyanun, M., Phusantisampan, T., 2016. Review: the bioavailability activity of *Centella asiatica*. Int. J. Appl. Sci. Technol. 9, 1–9.
Yemm, E.W., Willis, A.J., 1954. The estimation of carbohydrates in plant extracts by Anthrone. Biochem. J. 57, 508–514.