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Comparative studies on Turkish and Indian *Centella asiatica* (L.) Urban (gotu kola) samples for their enzyme inhibitory and antioxidant effects and phytochemical characterization

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ABSTRACT

In this study, enzyme inhibitory and antioxidant activity of the ethanol extracts of *Centella asiatica* (L.) Urban from Turkey and India was examined and the results were compared to those of the standardized extract of the plant obtained from China. Inhibitory activity of these three extracts was screened against four enzymes; acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase (TYRO), and lipoxygenase (LOX) using ELISA microplate reader. Antioxidant activity of the extracts was determined *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH), metal-chelation, and ferric-reducing antioxidant power (FRAP) assays. The extracts showed a notable inhibition against BChE and TYRO, while the extracts from Turkey and India displayed lower activity in the antioxidant assays. HPLC analysis indicated that the Turkish plant sample was found to be richest in *p*-hydroxy-benzoic acid, whilst the Indian sample contained chlorogenic acid in the most abundant amount. α-Copaene was the dominant compound in the essential oil of the plant sample from Turkey.

1. Introduction

Centella asiatica (L.) Urban (CA) (syn. *Centella coriacea* Nannfd., *Hydrocotyle asiatica* L., *Hydrocotyle lunata* Lam., *Trisanthus cochinchinensis* Lour.) (Apiaceae), known by several local names as "Gotu Kola, Asiatic pennywort, Indian pennywort, marsh penny ship rot, tiger herb", is a tropical edible plant growing naturally in Southeast Asian countries and has been used in ayurvedic medicine since centuries (Meulenbeld and Wujastyk, 2001). The preparations of the plant have been used in wound healing and memory enhancement and recorded for its proven biological effects in the monographs of European Pharmacopeia, Commission E belonging to German Ministry of Health, and World Health Organization (WHO) (Howes and Houghton, 2003). The extracts and some fractions of the plant have been also reported to possess several other medical uses against rheumatism, syphilis, leprosy, ulcer, and eczema (Somchit et al., 2004; Visweswari et al., 2010; Won et al., 2010), where mainly triterpenes and saponins, the largest chemical groups in *C. asiatica*, appear to be responsible for its therapeutic effects (Gohil et al., 2010). In addition to its biological effects, the plant is known to be consumed as leafy green vegetable in Sri Lanka and Philippines (Orhan, 2012). "Malluma", a traditional accompaniment to rice and curry, is prepared using *C. asiatica*, while the leaves are used for "sambai oi peuga-ga", a type of salad in Indonesia. The leaves are used for preparing a drink or can be eaten in raw form in salads or cold rolls in Vietnam, India, and Thailand. In Malay cuisine, the leaves of this plant are used for a type of Malay salad known as "ulam".

CA has been reported to contain triterpene derivatives in major amounts and the earliest examples of this compounds present in CA were identified in late 1940s as "asiatic acid and madecassic acid" along with their heterosides named as "asiaticoside" and "madecassoside" constituting approximately up to 10% of the plant (Orhan, 2012). Later on, compounds from various chemical classes have been also isolated such as flavonoids (Bhandari et al., 2007), polyacetylenes (Govindan et al., 2007), and phenolic acids (Subban et al., 2008).

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CA has been recorded to be used traditionally for its memory-enhancing effect, which is associated with Alzheimer's disease (AD). AD is a neurological disorder with a progressive nature characterized by cognitive deficit and behavioral abnormalities in the patient. It has become one of the deadly and costly diseases especially in the developed countries with increasing number of elderly population and the prevalence of the disease is expected to rise drastically in the next decades (Wilkinson et al., 2004). Although pathogenesis of AD has not been fully described, yet, "cholinergic" and "amyloid" hypotheses have been proposed and, therefore, cholinesterase inhibitors, in particular, have become the most prescribed drug class for the treatment of AD (Orhan et al., 2009). On the other hand, oxidative stress and iron dysregulation are important parameters, which can induce severity of AD (Tuppo and Arias, 2005; Mandel et al., 2007). Tyrosinase (TYRO), also known as polyphenol oxidase, is a copper-containing enzyme and associated with melanin synthesis, which may play a critical role in neuromelanin formation related to Parkinson's disease (PD) and cause TYRO-mediated dopamine neurotoxicity in the brain (Kumar et al., 2012). Hence, inhibition of TYRO might be beneficial for the patients diagnosed with PD (Asanuma et al., 2003). Moreover, many studies have proved that inflammation has accompanied in the pathology of neurological disorders including AD and PD (Kristofikova et al., 2013).

Taking traditional use of gotu kola for memory as starting point, we decided to undertake the current work in order to determine neurobiological effects of the ethanol extracts of the aerial parts of CA sample cultivated in Turkey for the first time, and a sample of the plant collected from its natural habitats in India through enzyme inhibition assays including acetylcholinesterase (AChE), butyrylcholinesterase (BChE) related to AD, tyrosinase (TYRO) linked to PD, and lipoxygenase (LOX) associated with inflammation mechanism as well as antioxidant assays. The selected phenolic acids were analyzed by HPLC in the ethanol extract of the plant, while essential oil composition of Turkish sample of CA was investigated by GC–MS for the first time herein.

2. Materials and methods

2.1. Plant materials

The aerial parts of CA cultivated in Turkey (CA-TR) were collected in June, 2010 from the medicinal plant garden belonging to Zeytinburnu Municipality in Istanbul (Turkey). The second sample of the plant growing naturally in India (CA-IND) was gathered in July, 2010 from Bangalore and identified by one of us (K.D.). The voucher specimens are preserved at Herbarium of Faculty of Pharmacy, Gazi University, Ankara (Turkey).

2.2. Extract preparation

The aerial parts of CA-TR and CA-IND were dried in shade at room temperature and ground in a mechanic grinder to fine powder and weighed accurately. The powdered plant materials were extracted with ethanol (75%) for three days. The ethanol phase of each plant material was filtered through regular filter paper and evaporated in reduced pressure until dryness to give the crude extracts, which were kept at +4 °C until the time bioactivity and phytochemical studies were performed. Yields (w/w) of the ethanol extracts were calculated as follows; CA-TR: 25.36% and CA-IND: 34.32%.

In addition, a commercially available standardized ethanol extract of *C. asiatica* (CA-STD) was obtained from Beijing Refine Biology Co., Ltd. (China) accompanied with the certificate of analysis, which indicated its standardization according to asiaticoside and madecassoside (10.78%) by HPLC.

2.3. Isolation of the essential oil of Turkish CA

The essential oil of the air-dried aerial parts of the plant from Turkey (CA-TR, 300 mg) was obtained by microdistillation apparatus (Eppendorf MicroDistiller®) to produce a small amount of essential oil which was trapped into *n*-hexane.

2.4. Enzyme inhibition assays

2.4.1. AChE and BChE inhibition

AChE and BChE inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman et al. (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5′-Dithiobis(2-nitrobenzoic) acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were same as described in our recent publication (Senol et al., 2010). Briefly, in this method, 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of test solution and 20 μL of AChE/BChE solution were added by multichannel automatic pipette (Eppendorf, Germany) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, Sunnyvale, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine, the anticholinesterase alkaloid-type of drug isolated from the bulbs of snowdrop (*Galanthus* sp.), was purchased from Sigma (St. Louis, MO, USA) and used as reference.

2.4.2. TYRO inhibition

Inhibition of tyrosinase (TYRO) (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with L-DOPA as substrate (Masuda et al., 2005). Assays were conducted in a 96-well microplate and an ELISA microplate reader (VersaMax Molecular Devices, CA, USA) was used to measure absorbance at 475 nm. The extracts dissolved in DMSO (50%) with 80 μL of phosphate buffer (pH 6.8), 40 μL of TYRO, and 40 μL of L-DOPA were put in each well. Each sample was accompanied by a blank that had all the components except for L-DOPA. Results were compared with a control consisting of 50% DMSO as well as the reference (α-kojic acid). TYRO inhibition (%) was calculated according to the formula given below:

$$\%\ \text{inhibition} = (\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})/\text{absorbance}_{\text{control}} \times 100$$

2.4.3. LOX inhibition

Inhibitory activity of the extracts against LOX was measured by modifying the spectrophotometric method developed by Tappel (1962). LOX (EC 1.13.11.12, type I-B), baicalein (reference drug), and linoleic acid (substrate) were purchased from Sigma (St. Louis, MO, USA). The reaction mixture of total volume of 190 μL contained sodium phosphate buffer (pH 8.0), sample and enzyme solutions, which were later incubated for 10 min at 25 °C. The