Antioxidant and DNA Damage Preventive Properties of Centella asiatica (L) Urb.

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ABSTRACT

Centella asiatica (L.) (Apiaceae) is commonly known as Mandukaparni. It is distributed in South America and all around Asia. It is a well known medicinal plant in Ayurveda system of medicine being used for various ailments like inflammation, diarrhea, asthma, tuberculosis, depression, memory loss and psoriasis. The aim of the present study was to assess the antioxidant potential in vitro of extracts of Centella asiatica in different solvents like hexane, chloroform, ethyl acetate, acetone, methanol and water. Highest polyphenols content was found in chloroform extract followed by methanol extract (9.04 µg/mg, 7.7 µg/mg gallic acid equivalents) and flavonoid content was found to be highest in water followed by chloroform extract (2.19 µg/g, 2.00 µg/g) respectively. The IC$_{50}$ value of the DPPH and hydroxyl radical scavenging activity of methanol extract showed 0.07 mg/ml and 500 µg/ml respectively. Reducing power assay results also followed in the same way. Methanol extract was comparatively effective in preventing more DNA damage. The results obtained in this study clearly indicate that C.asiatica has a significant potential as a natural anti-oxidant and DNA damage preventing agent.

Key words: Anti-oxidant; DPPH; Centella asiatica; DNA damage; Reducing power

INTRODUCTION

Medicinal plants have been a useful source for the research of new biologically active compounds. Different approaches are used to select a plant for research, specially the ethnomedical data approach. Apart from the medicinal effects of traditional herbs, exploratory researches have been made and a wide variety of new biological activities from traditional medicinal plants have recently been reported, including anticancer activity.[1]

Centella asiatica (L.) Urb., syn. Hydrocotyle asiatica (L.) popularly known in Brazil as Cairuçu-asiático, Centelha, Codagem and Pata-de-mula,[2] is a cosmopolitan member of the Umbelliferae family. In India, it is commonly known as ‘Mandukaparni’. In Srilanka and Indonesia it is given the name ‘Thankuni Sak’. In classical Indian Ayurveda literature, it is considered to be one of the ‘Rasayana’ (rejuvenator) drugs.[3] C. asiatica has also been reported to be useful in the treatment of inflammations, diarrhea, asthma, tuberculosis and various skin lesions and ailments like leprosy, lupus, psoriasis and keloid. In addition, numerous clinical reports verify the ulcer-preventive and antidepressive sedative effects of C. asiatica preparations, as well as their ability to improve venous insufficiency and microangiopathy.[4-5] Centella asiatica contains triterpene glycosides such as centellasaponin, asiaticoside, madecassoside, sceffoleoside,[6] asiatic acid and madecassic acid.[7-8] Asiaticoside is the most abundant triterpene glycoside in the water extract and it is transformed into asiatic acid in vivo by hydrolysis. Although the asiatic acid has shown cytotoxic activity on fibroblast cells[9] and induces apoptosis in different sorts of cancer.[10-14]

Free radicals are known as being capable of damaging a lot of cellular components such as proteins, lipids and DNA.[15] To protect the cells from oxidative damages by free radicals, produced during oxygen metabolism, an antioxidant system is used by aerobic organisms. The main antioxidant enzymes and agents such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione, ascorbic acid and tocopherol are important for cellular protection, due to their ability to eliminate free radicals, such as reactive oxygen species (ROS).[16]

Nowadays, there is an increasing interest in the biochemical functions of natural antioxidant extracts from vegetables,
fruits, and medicinal plants, which can become candidates to prevent oxidative damage, promoting health. The phenolic constituents found in vegetables have received considerable attention for being the main components of antioxidant activity, in spite of not being the only ones. The antioxidant activity of phenolic constituents has been attributed to its oxide-reduction properties, which play an important role in the adsorption or neutralization of free radicals.[17]

Based on its reputation to improve health condition, *Centella asiatica* has been selected for the present study and evaluated for its antioxidant properties and DNA damage protection using various in vitro systems.

**MATERIALS AND METHODS**

**Plant material**

*Centella asiatica* (L.) Urb. Leaves were collected from local market and identified with the help of herbarium collection, Department of Botany, University of Mysore, Mysore. The leaves were allowed to dry in shade for three days. The shade dried leaves were taken for further studies.

**Chemicals and reagents**

Hexane, chloroform, ethyl acetate, acetone, methanol and distilled water were used as solvent for extraction of antioxidant compounds. Other solvents and chemicals used in this study were dimethyl sulphoxide (DMSO), petroleum ether (40-60°C), H<sub>2</sub>SO<sub>4</sub>, NaOH, HCl, H<sub>3</sub>BO<sub>3</sub>, DPPH (1,1-Diphenyl-1-2-pircyl hydrazyl), BHA, Gallic acid, Folin-Ciocalteu reagent, FeCl<sub>2</sub>, ferrozine, potassium ferricyanide, NaEDTA, ascorbic acid, TCA, FeCl<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, catechin, deoxyribose, H<sub>2</sub>O<sub>2</sub>, thiobarbituric acid (TBA). All the chemicals and reagents were of analytical grade and were stored at prescribed conditions in the laboratory.

**Sequential extraction**

Fifty grams of crushed leaf sample was taken for extraction procedure. Cold extraction method was followed for sequential extraction of *centella* from non polar to polar solvents viz., hexane, chloroform, ethyl acetate, acetone, methanol and water. The extract was filtered through Whatman No.1. The filtrate was flash evaporated using a round bottom flask of known weight. Each of the extract was weighed and total yield was calculated for each solvent system. A known weight of each dried extract was dissolved in the respective solvents to prepare a stock solution of 100 mg/ml. All the stock solutions were kept at 4°C until further use.

**Total polyphenols estimation**

Total polyphenols content of each extract was determined using folin-ciocalteu reagent (FC reagent) method.[18] Briefly, sample in different amounts was mixed with distilled water to make up final volume (3 ml). Then, 0.5 ml FC reagent was mixed and incubated for 10 min at room temperature. Two milliliters of 7% Na<sub>2</sub>CO<sub>3</sub> was added and boiled the content in a boiling water bath for one minute. After cooling, absorbance was measured at 650 nm (there is no distinct absorbance maximum wavelength for polyphenols, so 650 nm wavelength was used). Gallic acid was used as a standard and amount of total polyphenols content was expressed as µg gallic acid equivalent per milligram (µg GAE/mg) extract.

**Total flavonoids estimation**

Estimation of total flavonoids by the method of Delcour and Varebeke.[19] Catechin was used as a standard and total flavonoids content was expressed as microgram catechin equivalents per milligram (µg CE/mg) extract. In brief, sample volume was make up with methanol to 1 ml. Then, 5 ml of Chromogen reagent (HCl + CH<sub>3</sub>OH + Cinnamaldehyde in 1: 3: 0.004 ratio) was added to each test tube and absorbance was measured at 640 nm.

**DPPH radical scavenging activity**

The antioxidant activity of different extracts was checked on the basis of 1, 1 diphenyl–2–picrylhydrazyl (DPPH) free radical scavenging activity. DPPH assay was performed as per the method described by Eberhardt et al.,[20] DPPH (500 µl, 0.5 mM in methanol) solution was mixed with different amounts of sample and volume was made to 3.5 ml with methanol. The mixture was incubated in dark for 45 min at room temperature. Absorbance was recorded at 515 nm in a spectrophotometer. BHA was used as standard antioxidant compound. A positive control was prepared by mixing 3 ml of methanol and 0.5 ml of DPPH solution. Sample blanks were prepared in methanol without DPPH solution to eliminate the absorbance of crude extracts. Methanol was used as blank. The DPPH radical scavenging activity percentage was calculated by using the formula as given below:

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\text{DPPH}^\circ \text{ scavenging activity (％)} = \left( \frac{A_c - A_s}{A_c} \right) \times 100
\]

Where \(A_c\) is the absorbance of positive control solution and \(A_s\) is the absorbance of test solution. IC<sub>50</sub> value, the concentration of sample or extract required to scavenge 50% of the DPPH free radicals in the mixture, was calculated using a linear regression equation derived from the graph of % DPPH scavenging activity and sample concentration.

**Hydroxyl radical scavenging activity**

Deoxyribose degradation assay was performed as per the method of Halliwell et al.,[21] with slight modification. Briefly, different concentrations of extracts were mixed with 200 mM FeCl<sub>2</sub> and 1.04 mM EDTA (0.2 ml, 1:l), 1 mM H<sub>2</sub>O<sub>2</sub> (0.1 ml), 28 mM deoxyribose (0.1 ml) and 1 mM ascorbic acid (0.1 ml) and the final volume was made to 1.1 ml with phosphate buffer (0.2 mM, pH 7.2). The mixture was...

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Total polyphenols and Flavonoids

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Polyphenolic compounds have a wide range of protective effects such as anti-inflammatory responses, prevention of low density lipoprotein oxidation and anti-hypertensive, anti thrombic and carcinostatic actions.

In this study highest polyphenols content was found in chloroform extract of C.asiatica followed by methanol extract, followed by water (9.04 μg/mg, 7.7 μg/mg, 6.76 µg/mg Gallic acid equivalents respectively) (Figure-1).

DNA damage protective activity

DNA damage protective activity of Centella asiatica was checked using pRSETA plasmid grown in E. coli. Plasmid DNA was isolated using QIA prep Spin Mini prep kit. Plasmid DNA was oxidized with H2O2 + UV treatment in presence of plant extract (PE) and checked on 1% agarose gel according to Russo et al.,[23] with minor modifications. In brief, the experiment was performed in a 10 µl volume in a micro centrifuge tube containing 200 ng of pRSETA plasmid DNA in TE buffer (10 mM Tris-Cl and 1 mM EDTA) pH 8.0. H2O2 was added at final concentration of 10 mM/ml with various concentrations of plant extract (1-3 µl of 10 mg/ml concentration). The reactions were initiated by UV irradiation and continued for 5 min on the surface of a UV transilluminator (312 nm) under room temperature. After irradiation the reaction mixture along with gel loading dye (6X) was loaded on to 1% agarose gel and run at 200 V for 1 hr. Untreated pRSETA plasmid DNA was used as a positive control in each run of gel electrophoresis.

RESULTS AND DISCUSSION

Plant biochemicals are gift from nature and antioxidant compounds are typical representative of these botanical gifts. Antioxidants are the substances which can protect the human body from free radicals and the ROS effects and retard the progress of many chronic diseases.[24-25] Apart from their biological functions in plants, these antioxidants are widely present in food products and agricultural raw materials. As the name antioxidant indicates that these compounds participate in oxidation-reduction processes, which have complex reaction mechanisms, so there is no single testing method capable of providing a comprehensive picture of the antioxidant profile of a studied sample. Many compounds in food have antivirus properties, which are capable of interacting with reactive molecules. Polyphenols and flavonoids are the powerful antioxidants within bioactive constituents.

Total reducing power estimation

The total reducing power of different extracts was determined following the method of Oyaizu.[22] Different amount of extract was mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture followed by centrifugation at 3000 rpm for 10 min. A 2.5 ml portion of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl3. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

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Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties.[27] Therefore, it is valuable to determine the total flavonoids content of the extracts under study. As shown in Figure-2, maximum amount of flavonoids was found in water followed by chloroform fractions, respectively. Several studies have shown that many flavonoids contribute significantly to the total antioxidant activity of plants. There is abundant evidence that flavonoids are effective in blocking oxidant induced neuronal injury.[28]
**Antioxidant activity**

Determination of antioxidant activity of different solvent extracts of *Centella* was based on DPPH radical scavenging activity and Hydroxyl radical scavenging activity (TBARS). The antioxidant activity of different extracts was calculated as their capacity to scavenge free radicals of DPPH, which

![Graphs showing DPPH radical scavenging activity of different extracts of *C. asiatica*.](image)

**Figure 3:** DPPH radical scavenging activity of different extracts of *C. asiatica*

![Graph showing Hydroxyl radical scavenging activity of different extracts of *C. asiatica*.](image)

**Figure 4:** Hydroxyl radical scavenging activity of different extracts of *C. asiatica*

has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources.\[^{29}\]

Although radical scavenging activity should not be considered as being synonyms with antioxidant activity, it is a fact that almost all of the powerful natural antioxidants, such as tocopherol, carnosal and ascorbic acid are also strong scavengers of the DPPH radical. Results depicted in Figure-3 showed DPPH activity of sequential extracts of Centella (Hexane, Chloroform, Ethylacetate, Acetone, Methanol and Water respectively). Methanol extract was observed with highest percentage of inhibition of free radicals with IC\(_{50}\) values (0.07 mg/ml), while hexane fraction is least potent. The highest free radical scavenging activity of methanol extract can be attributed to the presence of polyphenols and flavonoids as this fraction contains maximum amount of these secondary metabolites.

Hydroxyl radicals are most reactive ROS, capable of attacking most of the biological substrates. The prevention of such deleterious effect is very necessary in terms of both human health and the shelf-life of food, cosmetics and pharmaceuticals. Therefore, it was considered important to assess the protective ability of the sample extract against OH radicals. In the OH radical – mediated 2-deoxy-D-ribose degradation assay, OH radicals are generated by Fenton chemistry using EDTA, Fe\(^{2+}\) ions, ascorbic acid and H\(_2\)O\(_2\). Six sample extracts (Hexane, Chloroform, Ethyl acetate, Acetone, Methanol and Water) were taken for estimation of OH radicals scavenging activity. IC\(_{50}\) values were calculated using linear regression equations. Methanol extract showed highest hydroxyl radical scavenging activity (IC\(_{50}\)=500 µg/ml). Graphical presentation of the data has been shown in Figure-4. Ferric ion reduction to ferrous ion reducing power was observed more in methanol extract followed by water extract (Figure-5).

**DNA damage protective activity**

DNA damage protection studies were performed using methanol and water extracts. Protection of DNA strand breaks was observed more in methanol extract followed by water extract showed protection of supercoiled plasmid is directly proportional to the activity of plant extract (Figure-6).

The faster moving prominent band (lane. 1) corresponded to the native supercoiled circular DNA (Sc DNA) and the slower moving very faint band was the open circular form (Oc DNA). The UV irradiation of DNA in the presence of H\(_2\)O\(_2\) (lane. 2) resulting the cleavage of Sc DNA to give prominent Oc DNA and a faint linear DNA (Lin DNA) indicating that OH- generated from UV-photolysis of H\(_2\)O\(_2\) produced DNA stand scission. The C.asiatica extract was able to prevent this damage to a large extent.

**CONCLUSIONS**

Results of the present study prove that methanolic extract of C.asiatica possess very pronounced biological properties such as anti oxidant activity, reducing power and DNA damage protection activity. Furthermore these results of total polyphenols and flavonoids presence in hexane and methanolic extracts strengthen the biological activity.

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