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In-vitro Anti-Oxidant Property of *Vallarai (Centella asiatica)* cultivated by conventional and traditional Methods

Research Article

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Abstract

In ancient times, Siddhars have been described and also used the traditional methods to cultivate the anti-oxidant herbs for rejuvenation purpose and also they are listed some plants as anti-oxidant herbs. They were used *Semicarpus anacardium* manure for cultivation of anti-oxidant plants. In Siddha, these anti-oxidant herbs are mentioned as *Kayakarpam* (Rejuvenation therapy). Most of the Non Communicable Diseases (NCD) are caused by oxidative stress. These anti-oxidant herbs are helps to reduce oxidative stress and prevent the incidence of NCDs. *Centella asiatica (Vallarai)* is one of the *Kayakarpa* medicinal plants. The traditional cultivation method for *Kayakarpa* herbs are also described in Siddha especially for *Centella asiatica* (*Vallarai*). Therefore, this study was aimed to validate the *in-vitro* anti-oxidant property of *Vallarai* cultivated by conventional (sample A) and traditional methods (sample B). The study samples were screened for anti-oxidant activity by DPPH assay, Nitric Oxide radical scavenging assay, ABTS assay and H_2O_2 radical scavenging assay and the IC_{50} value of the study drug, sample-A was 88.6 ± 8.536 (µg /ml), 183 ± 15.55 (µg/ml), 78.92 ± 8.43 (µg /ml) and 183 ± 11.64 (µg /ml) whereas sample- B was 57.06 ± 1.221 (µg /ml); 145.1 ± 13.12 (µg/ml); 96.45 ± 3.966 (µg /ml) and 132.3 ± 18.71 (µg /ml) respectively. This study results revealed that, both samples are possessing anti-oxidant property and sample B have greater scavenging activity compared to sample A. Hence, The Traditional method can be adopted for cultivation of anti-oxidant herbs, which helps to retain the active components and enhances the anti-oxidant potency of medicinal herbs.

Key Words: *Kayakarpam,* Anti-oxidant, *Centella asiatica,* Traditional cultivation method.

Introduction

Most of the Non Communicable Diseases (NCD) are caused by oxidative stress, inflammation and mitochondrial alterations. The anti-oxidant herbs or substances are help to reduce oxidative stress and prevent the NCDs like cardiovascular diseases, neurodegenerative diseases, cancer, diabetes and obesity etc. (1). Natural resources are having rich amount of anti-oxidant property. It act as a radical scavengers and helps in rejuvenation therapy. In Siddha, anti-oxidants are mentioned as *Kayakarpam* (2) (Rejuvenation therapy). In ancient times, Sage Siddhars have been listed some anti-oxidant herbs and also they described the traditional cultivation methods for rejuvenation purpose by using *Semicarpus anacardium* manure for cultivation (3). Siddhars used the *Kayakarpam* for the long living through preventing the diseases and treating the acquired diseases. *Thirumoolar* stated that in *Thirumanthiram*, the *Kayakarpam* protects the body and it acts as anti-oxidants which reduce the oxidative stress, cell damage and prevents aging by scavenging the free radicals.

Free radicals and reactive oxygen specious (ROS) are capable of damaging the important macromolecules like proteins, lipids etc. and leading to cell damage and disruption of homeostasis. Free radicals involve in the development of cancer, heart diseases and also aging. Oxidative stress is simply the elevation of free radicals found in cells that accumulate to higher than normal levels. Excessive oxidative stress damages cells and tissues, specifically mitochondria, cell membranes, DNA, proteins, and lipids (4). Oxidative stress takes place naturally and plays an important role in process of aging. Antioxidants prevent free radical induced tissue damage by preventing the formation as well as accumulation of radicals, scavenging them, or by promoting their decomposition and impede the progress of chronic diseases (5). The usage of anti-oxidant was emerging in the global market as anti-aging or rejuvenators in the form of face creams, body lotions and hair oils etc. But in ancient times Siddhars have been practiced the anti-oxidant herbs, rejuvenating activities like Yogam, Pranayamam in day-to-day life. *Centella asiatica (Vallarai)* is one of the *Kayakarpa* medicinal plants. The traditional cultivation method for *Kayakarpa herbs* are also described in

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Siddha (3) especially for *Centella asiatica* (*Vallarai*). Commonly people are using this plant for rejuvenation and to enhance the memory (Brain Booster) (6).

Centella asiatica (L.) Urban, a tropical medicinal plant belonging to the family Apiaceae, is widely distributed in many parts of the world especially in Asian countries such as India, China, Nepal, and other Southeast Asia countries including Malaysia and Indonesia. The plant can also be found in Australia, South Africa, the United States, and Europe (7). Major phytochemical present in *Centella asiatica (Vallarai)* are triterpenoid saponins, madecassoside, asiaticoside, asiatic acid, madecassic acid and minor phytochemicals are asiaticoside B, brahminoside, brahmoside, centelloside, indcentelloside, thankuniside, isothankuniside, brahmic acid, isobrahmic acid, betulic acid, centic acid and centoic acid (8).

Centella asiatica has astringent, bitter and sweet taste, coolant potency and which is indicated for gastric ulcer, diarrhoeal diseases, elephantiasis, etc. (6) and used for the preparation of *Vallarai Nei (9), Vallarai ennai* (6). It is also used in insomnia, cardiac debility, epilepsy, laryngitis, asthma, bronchitis, hiccough, abdominal disorders, leprosy, strangury and fever. It has been found to be useful in diseases of skin, nerves and blood. Leaves are useful in abdominal disorders due to dysentery in children. They are also used as tonic and for improving memory, useful in syphilitic skin diseases both internally and externally (10). According to Siddha Materia medica *Vallarai* has Alterative, Tonic, Diuretic, Stimulant, Emmenagogue (6), and scientifically it was proved for its cardio protective effect (11), neuroprotection and cognitive enhancement action (12), anti-hyperglycemic activity (13), anti-cancer and anti-oxidant activity (14).

The study was aimed to validate the *in-vitro* anti-oxidant property of *Centella asiatica* cultivated by conventional and traditional methods. So that, this type of study may help to promote the traditional methods of cultivation for *Kayakarpa* medicinal plants.

Materials and Methods

The study samples were collected from two cultivation fields. Two fields were selected for the cultivation process with same quality of soil and surroundings, one for conventional method and another one for traditional cultivation method.

Preparation of *Semicarpus anacardium* manure (3)

The *Semicarpus anacardium* (*Serankottai*) seeds were cut into small pieces and spread in the prepared field and it was covered with sand, which was well irrigated continuously for 3 months until the entire *Semicarpus* nuts were decomposed with soil.

Plantation of *Centella asiatica (Vallarai)*

The seedlings of *Centella asiatica (Vallarai)* were planted in normal land (Conventional method) and in fertilized land (Traditional method) and irrigated well.

Collection of samples

After 2 months of planting, the leaves of *Centella asiatica (Vallarai)* from both lands were collected and dried in room temperature and named as Sample A and Sample B respectively.

Anti-oxidant activity

The collected samples were subjected to evaluate the anti-oxidant activity by using *in-vitro* anti-oxidant assay methods.

DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) assay: (15)

The antioxidant activity of test drug samples A and B were determined using the 2, 2-diphenyl 1-2 picrylhydrazyl (DPPH) free radical scavenging assay method. The test Sample was mixed with 95% methanol to prepare the stock solution in required concentration. From the stock solution 1ml, 2ml, 4ml, 6ml 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent was made the final volume of each test tube up to 10 ml whose concentration is then10 µg/ml, 20 µg/ml, 40µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml respectively. Ascorbic acid was used as standard and it was prepared in same concentration as that of the sample extract by using methanol as solvent. Final reaction mixture containing 1 ml of 0.3 mM DPPH methanol solution was added to 2.5 ml of sample solution of different concentrations and it was allowed to react at room temperature. Absorbance in the presence of test sample at different concentrations (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100µg/ml) were noted after 15 min incubation period at 37^{0}C. Absorbance was read out at 517 nm using double-beam U.V Spectrophotometer by using methanol as blank.

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

The effective concentration of test samples required to scavenge DPPH radical by 50% (IC_{50} value) were obtained by linear regression analysis of dose-response curve plotting between % of inhibition and concentrations.

Nitric Oxide Radical Scavenging Assay: (16)

The concentrations of test samples were made into serial dilution from 10−100 µg/mL and the standard Gallic acid. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the test drugs (10−100 µg/mL) and incubated at 25°C for 180 mins. The test drugs were mixed with an equal volume of freshly prepared Griess reagent. Control samples without the test drugs but with an equal volume of buffer was prepared in a similar manner as was done for the test samples. The absorbance measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader.

Gallic acid used as the positive control. The percentage of inhibition of the test drugs and standard were calculated and recorded. The percentage of nitrite radical scavenging activity of the test drugs and Gallic acid was calculated by using the following formula:

$$\text{Nitric oxide scavenged (\%)} = \frac{\text{A control- A test}}{\text{A control}} \times 100$$

Where A control = absorbance of control sample and A test = absorbance in the presence of the samples extracts of standards.

ABTS Assay: (17)

This assay carried out for the purpose of evaluating the anti-oxidant potential of test drugs against 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals. The ABTS radical cation method was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture then kept in the dark at room temperature for 16 h to allow free radical generation and then diluted with water (1 : 44, v/v). To determine the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of test sample (10-100µg/ml) and incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control. Gallic acid with same concentrations of test drugs were measured following the same procedures described above and used as positive controls. The antioxidant activity of the test samples were calculated by using the following equation:

$$\text{Radical scavenging (\%)} = \frac{\text{(A) Control - (A) sample}}{\text{(A) Control}} \times 100$$

Hydrogen Peroxide Radical Scavenging Assay: (18)

A hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 mL) of the test sample A and B (different concentration ranging from 10-100µg/ml) were transferred into the test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After adding 0.6 mL hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. Butylated hydroxyanisole (BHA) was used as the positive control. The percentage of inhibition of the test sample A, B and standard were calculated and recorded. The percentage radical scavenging activity of the test sample A and B and BHA was calculated by using the following formula:

$$\text{Radical scavenging (\%)} = \frac{\text{(A) Control - (A) sample}}{\text{(A) Control}} \times 100$$

Results and Discussion

In DPPH radical scavenging assay, the trial drugs sample - A and sample - B showed that the percentage of inhibition ranges from 7.746 ± 1.797 to 53.79 ± 3.626 for sample A and 16.47 ± 2.983 to 74.7 ± 1.731 % for sample B when compared with standard drug Ascorbic acid with percentage of inhibition ranges from 45.28 ± 3.244 to 97.08 ± 1.24 %. The IC_{50} value of the trial drug was 88.6 ± 8.536 (µg /ml) for sample A and 57.06 ± 1.221 (µg /ml) for sample B when compared with standard drug ascorbic acid with IC_{50} value 15.13 ± 2.464 µg/ml. Sample B shows higher DPPH radical scavenging activity than sample A.

Figure 1: DPPH radical scavenging activity of *Centella asiatica*

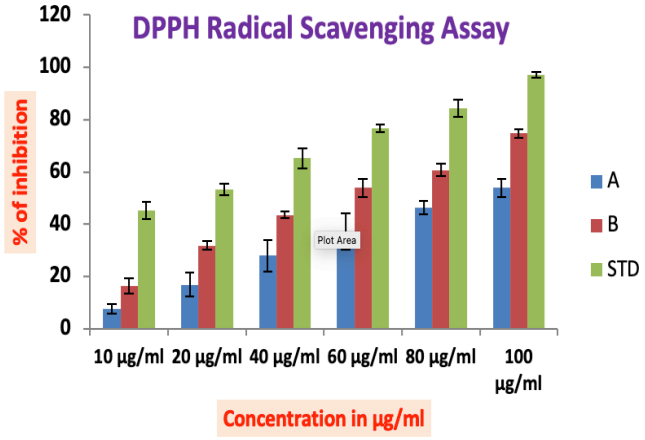

Table 1: IC_{50} Values for DPPH radical scavenging activity of *Centella asiatica*

Test Drug / Standard	IC_{50} Value DPPH Assay ± SD (µg /ml)
Ascorbic acid	15.13 ± 2.464
Sample A	88.6 ± 8.536
Sample B	57.06 ± 1.221

Data are given as Mean ± SD (n=3)

In Nitric Oxide radical scavenging assay, the result revealed that the percentage of inhibition of the test drugs sample - A and ample - B ranges from 7.971 ± 4.641 to 30.46 ± 1.885 for sample A and 17.07 ± 3.84 to 39.26 ± 0.8682 % for sample B when compared with standard drug Gallic acid with percentage of inhibition ranges from 44.22 ± 8.589 to 97.76 ± 0.7092 %. The IC_{50} value of the trial drug was 183 ± 15.55 (µg/ml) for sample A and 145.1 ± 13.12 (µg/ml) for sample B when compared with standard drug Gallic acid with IC_{50} value 12.85 ± 10.36 µg/ml. On comparing both IC_{50} values, Sample B has effective radical scavenging activity than sample A.

Figure 2: Nitric Oxide Radical Scavenging activity of *Centella asiatica*

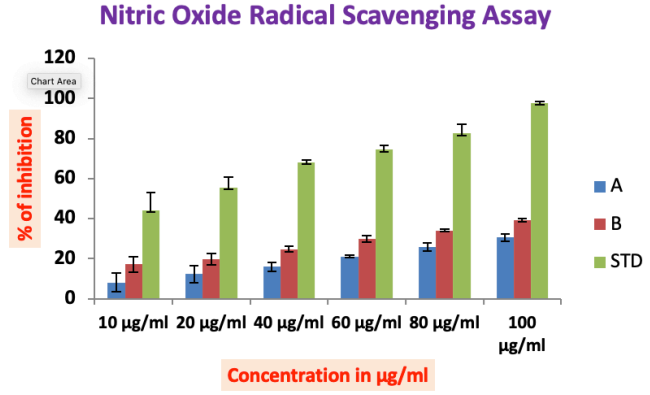

Table 2: IC_{50} Values for Nitric Oxide radical scavenging activity of *Centella asiatica*

Test Drug / Standard	IC_{50} Value NO Assay ± SD (µg /ml)
Gallic acid	12.85 ± 10.36
Sample A	183 ± 15.55
Sample B	145.1 ± 13.12

Data are given as Mean ± SD (n=3)

In Hydrogen peroxide radical scavenging assay, the trial drugs sample - A and sample - B showed that the percentage of inhibition ranges from 2.94 ± 0.5557 to 27.03 ± 2.569 for sample A and 9.888 ± 2.34 to 39.59 ± 4.583 % for sample B when compared with standard BHA with percentage of inhibition ranges from 33.39 ± 2.94 to 90.68 ± 4.421 %. The IC_{50} value of the trial drug was 183 ± 11.64 (µg /ml) for sample A and 132.3 ± 18.71 (µg /ml) for sample B when compared with standard BHA with IC_{50} value 38.03 ± 1.634 µg/ml. The results revealed that the sample B shows higher Hydrogen peroxide radical scavenging activity than sample A.

Fig.no:3. Hydrogen peroxide radical scavenging activity of *Centella asiatica*

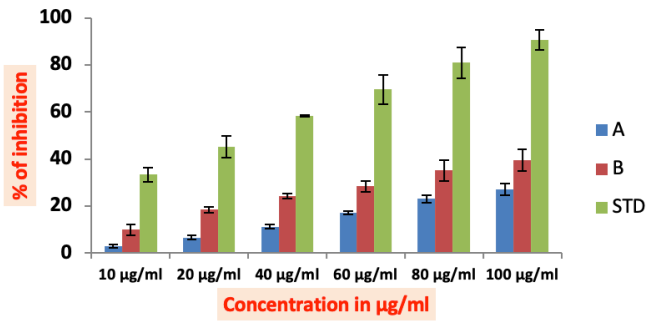

Table 3: IC_{50} Values for Hydrogen peroxide radical scavenging activity of *Centella asiatica*

Test Drug / Standard	IC_{50} Value Hydrogen peroxide radical scavenging Assay ± SD (µg /ml)
Sample A	183 ± 11.64
Sample B	132.3 ± 18.71
BHA	38.03 ± 1.634

Data are given as Mean ± SD (n=3)

In ABTS radical scavenging assay, the trial drugs sample - A and Sample - B showed that the percentage of inhibition ranges from 14.2 ± 0.3889 to 54.16 ± 7.832 for sample A and 3.259 ± 0.974 to 49.32 ± 1.317 % for sample B when compared with standard drug Gallic acid with percentage of inhibition ranges from 31.32 ± 3.909 to 96.39 ± 1.535 %. The IC_{50} value of the trial drug was 78.92 ± 8.43 (µg /ml) for sample A and 96.45 ± 3.966 (µg /ml) for sample B when compared with standard drug Gallic acid with IC_{50} value 24.25 ± 3.571µg/ml. The results revealed that the sample A possess higher ABTS radical scavenging activity than sample B.

Fig. No: 4. ABTS radical scavenging activity of *Centella asiatica*

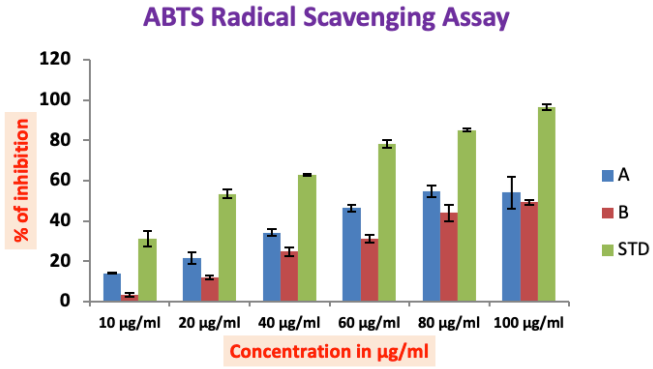

Table 4: IC_{50} Values for ABTS radical scavenging activity of *Centella asiatica*

Test Drug / Standard	IC_{50} Value ABTS Assay ± SD (µg /ml)
Sample A	78.92 ± 8.43
Sample B	96.45 ± 3.966
Gallic acid	24.25 ± 3.571

Data are given as Mean ± SD (n=3)

Conclusion

The *In-vitro* anti-oxidant activities were studied for *Centella asiatica* sample A - (cultivated by conventional method) and sample B (cultivated by traditional method). This study results revealed that the both samples of *Centella asiatica* possess good anti-oxidant property but sample B exhibited higher scavenging activity when compared to sample A. The outcome of the study may helps to cultivate the medicinal herbs by traditional cultivation method for rejuvenation purpose. This cultivation method can be adopted for conservation of endangered herbs also, which helps to retain the active components. This is a preliminary study and further studies will be carried out to explore the value and importance of traditional cultivation methods.

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