

Neuroprotective properties of Madecassoside from *Centella asiatica* after hypoxic-ischemic injury

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Abstract: Madecassoside is one of increasingly used constituent of *Centella asiatica*, a frequently prescribed crude drug in South eastern Asia and China for wound healing. In the present experiment, it exposes the neuroprotective nature of Madecassoside in GT1-7 cell lines, further, which the antioxidant activities are performed. The cellular toxicity was assessed using 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay with increased cell viability with IC₅₀ 2.5µg/ml. the regulation of antioxidant levels showed changes in madecassoside treated cell lysate viz., SOD assay. Also, the antioxidative assays confirmed the negligible cellular damage caused to the GT1-7 cell lines. Hence, the results advocate that the current antioxidant and antitumor activity be justified by the high concentration of phenolic constituents, primarily the triterpene present in the *C. asiatica*.

Keywords: GT1-7, cell lines, Madecassoside, DPPH, MTT assay, LDH.

INTRODUCTION

Centella asiatica (L.) Urban (Family-Apiaceae), an increasingly used medicinal herb traditionally employed as a wound healing factor in South Asian and European countries (James and Dubery, 2009; Thong-On *et al.*, 2014). *C. asiatica* contains triterpene glycosides like asiaticoside, madecassoside (Inamdar *et al.*, 1996). Also, it has the anti-diabetic property of *C. asiatica* for centuries that are following Ayurvedic system of medicine (Rahman *et al.*, 2012). Its storage-enhancing effect, which is associated with Alzheimer's disease. Madecassoside, a triterpenoid, is a major component of *Centella asiatica*, which is a traditional herb, used in Asiatic countries. It has been employed as a wound-healing agent and for the prevention of cicatrization, because it has been proven to effectively promote fibroblast proliferation and collagen synthesis (Sampson *et al.*, 2001; Cao *et al.*, 2010).

Hypoxic ischemia is a usual case of impairment to the neonatal brain. The systemic and cerebrovascular physiologic factors play a significant part in the initial phases and the intrinsic vulnerability of specific cell types and systems in the growing brain may be more significant in shaping the final form of damage and functional disability (Johnston *et al.*, 2001). The cerebrovascular factors contribute to the pathophysiology of hypoxic ischemic brain injury and have played an dominant function in is a growing awareness among clinicians based on the use of MR brain imaging that Hypoxic and Ischemic Encephalopathy (HIE) targets special brain structures (Johnston, 1998). Subsequently, in the present study the experimentation are to make a relationship the

antioxidant tests viz., total phenolic content and antioxidant activity with cytotoxic action coming about because of the neuroprotective properties of madecassoside on the GT1-7 neural growth cell lines.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), and 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) were received from Sigma Chemical Co. (St. Louis, MO). The compound Madecassoside (MA) was purchased from Sigma-Aldrich (Sigma-Aldrich Shanghai Trading Co Ltd).

Antioxidant activity

DPPH radical scavenging activity

Using the scavenging action of 2,2-diphenyl-1-picrylhydrazyl radicals, the scavenging activity of DPPH was assessed according to Parejo *et al.*, (2000). 0.3mM DPPH was made by dissolving the DPPH in methanol was made as stock solution. 100µl DPPH solution was added to 100µL of the lysate from concentrations (10-500µg/mL) and vibrated vigorously. Water and DPPH was kept as control and the experiments were carried in triplicates and were averaged. The DPPH scavenging activity was calculated using:

Percentage inhibition (%) = [(Control absorbance - Sample absorbance) / (Control absorbance)] x 100

Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent as per Guha *et al.*, (2010).

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Different concentrations were made by diluting cell lysate in methanol ranging from (125, 250, 500 and 1000 μ g/ml). The control sodium carbonate solution (2mL of 7.5% Na₂CO₃ in 2.55 cc of distilled water) was used as blank with absorbance at 765nm. The results were expressed as Gallic acid equivalence in μ g.

Super oxide dismutase assay (SOD)

Super oxide dismutase (SOD) were assayed by utilizing the nitrobluetetrazolium (NBT) method of Beauchamp and Fridovich (1971). NBT was reduced to the blue formation by O₂⁻, which bears a strong absorbance at 560 nm. The absorbance of formation was then quantified in the supernatants.

Lactate dehydrogenase assay (LDH) leakage assay

The leakage of LDH was measured using LDH kits (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's protocol. The ratio of LDH activity in the culture medium to the total LDH activity in the cell lysate and medium and detected at the wavelength of 450nm.

Cell lines

Hypothalamic Neuronal Mouse Cell Lines (GT1-7 cells) from American Type Culture Collection (ATCC) were made in the high-glucose Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, Shanghai, China), penicillin G (100U/ml), 1% (v/v) L-glutamine, and streptomycin (100mg/cc) in a humidified 5% CO₂ atmosphere at 37°C. Cells in exponential growth were detached with 0.25% trypsin-0.02% EDTA (Solarbio, Beijing, China) and were seeded in a 96-well plate at a seeding density of 3-4 $\times$ 10⁴ /well before being incubated for 24 h as described by (Mellon *et al.*, 1990; Mirshamsi *et al.*, 2004). To induce hypoxia (deprivation of oxygen supply), the cell cultures were placed in CO₂ incubator (Billups Rothenberg, Del Mar, CA) with 5% CO₂ (control) and humidified 95% N₂/5% CO₂ (hypoxia), for 24h at 37°C (Koretz *et al.*, 1994).

Cytotoxicity

Cytotoxicity was assessed by evaluating the cell viability after treatment with madecassoside using MTT method. The cell at exponential growth were sown at a seeding density of 4 $\times$ 10⁵/well in a 96-well plate and covered overnight to allow adhesion. The cells pre-treated with different concentrations of madecassoside ranging starting from 0.5, 1, 2.5, 5 and 10 μ g/ml, after treatment, the MTT solution (2.5mg/mL) was added to each well to establish a final immersion of 0.25mg/ml and the plates were nurtured for 4h at 37°C. The supernatant was removed, and the metabolic product-formazan, was resuspended in DMSO and perceived at 490nm on a plate reader. Cell viability (%) was calculated as the absorbance of each injured group divided by the absorbance of the normoxic control multiplied by 100 as per Mosmann (1983).

STATISTICAL ANALYSIS

Results were expressed as mean $\pm$ SD. Statistical analyses were done with one-way ANOVA followed by Dunnett's test to find significant differences ($P < 0.05$) among the group. Trials were held out in triplicates. Antioxidant activities and cell viability were compared with that of control. The average values were estimated from the triplicate values. Information from the test groups were compared with SC₅₀ value of the measure which is the concentration of sample, required to scavenge 50% of DPPH free radicals.

RESULT

The cytopathic effect (CPE) was made under an inverted microscope. The dilution causing microscopically detectable alteration of normal cellular morphology of the confluent cell lines were evaluated at 50% CPE with IC₅₀ 2.5 μ g/mL (fig. 1). In the present work, we have estimated total phenol, SOD, and DPPH in the madecassoside against the GT1-7 cell neural lines. The antioxidative activity of plant parts is mainly given by the active compounds present in them. This is particularly true for madecassoside a main portion of leaf extracts of *C. asiatica*, which had exceptionally high antioxidative activities that were not significantly ($P < 0.05$) different from that of ascorbic acid. Others have also described, that the total phenolic content of the tested vegetable extracts was correlated with the DPPH activity, indicating that total phenolics can play a major part in the antioxidant activity of plant materials (Zhou and Yu, 2006).

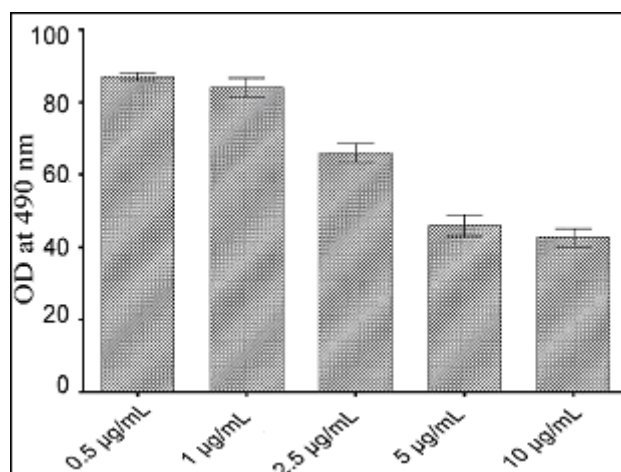


Fig. 1: The cytopathic effects of Madecassoside on GT1-7 neural cell lines. Results are represented as mean $\pm$ standard deviation, n=3. Error bars are standard error of mean. Statistical significant differences ($P < 0.05$).

The total phenol content of cell lysate containing madecassoside, which were examined at 1 μ g/mL against Gallic acid (GA). The GA equivalents of the estimated

phenolic concentrations ranged from 0.327 ± 0.110 to $0.572 \pm 0.023 \mu\text{g/GA}$ as shown in fig. 2, signifying the presence of polyphenols. The madecassoside treated cell lysate was subjected to SOD assay, which remarkably shown regulation of antioxidant levels (fig. 3).

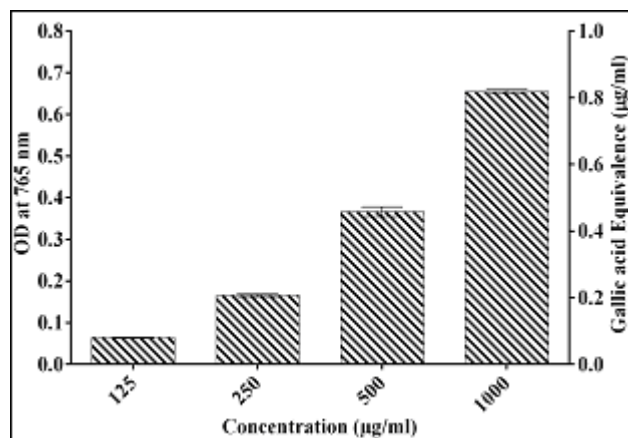


Fig. 2: Effect of madecassoside on total phenol content assay. Results are represented as mean $\pm$ standard deviation, $n=3$. Error bars are standard error of mean. Statistical significant differences ($P<0.05$).

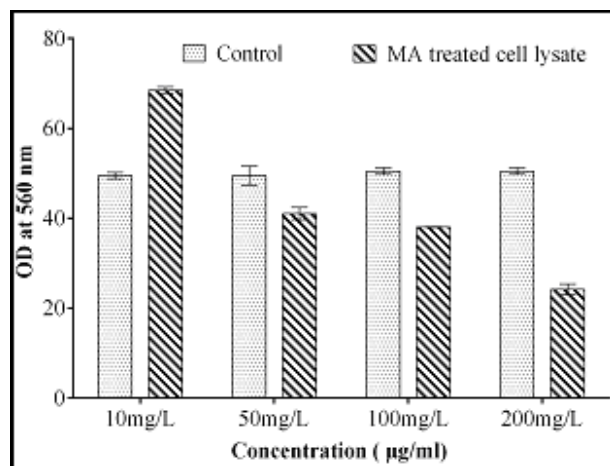


Fig. 3: Antioxidant activity of madecassoside assayed by super oxide dismutase method. Results are represented as mean $\pm$ standard deviation, $n=3$. Error bars are standard error of mean. Statistical significant differences ($P<0.05$).

The antioxidant potency of the madecassoside treated cell lysate and DMSO were measured by DPPH radical scavenging action. The outcomes are shown as % inhibition of DPPH and reported in fig. 4. Ascorbic acid showed a higher activity with scavenging activity from a concentration of 1.25 mg/cc . The radical scavenging activity of the madecassoside on DPPH showed high activity with $<4.5 \mu\text{g/ml}$. DPPH radical scavenging was found to be proportional to the doses. Earlier, *C. asiatica* leaves have been reported to possess DPPH radical scavenging activity with IC_{50} values $41.36 \mu\text{g/ml}$.

The protective effects of the *C. asiatica* against the hypoxia were further confirmed through examining their effects on the LDH leakage. The escape of electrons from the cells was evaluated as the percentage of super oxide dismutase activity in the cultivation medium. LDH leakage can provide data on the cell membrane damage or cell death but not the nature of cell death (fig. 5). Whereas, the pretreatment of GT1-7 cells with varying degree of concentrations madecassoside markedly protected the cells from hypoxia injury, with effects better than or comparable to that of the positive control N-acetyl-L-cysteine whose neuroprotective effect was also supported. The substantial reductions in LDH leakage also supported the neuroprotective effects of madecassoside compound.

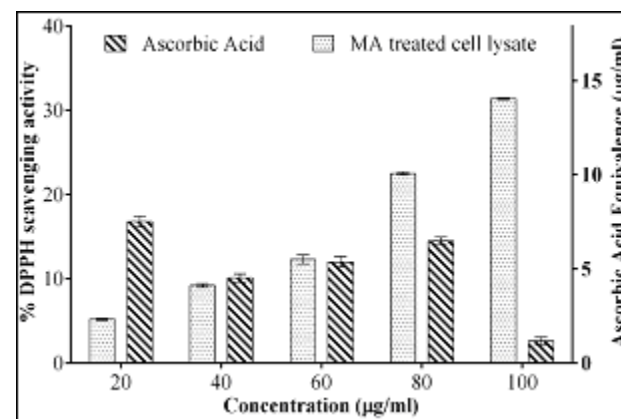


Fig. 4: Antioxidant potential of the madecassoside were measured by DPPH radical scavenging activity. Results are represented as mean $\pm$ standard deviation, $n=3$. Error bars are standard error of mean. Statistical significant differences ($P<0.05$).

DISCUSSION

Antioxidative properties of essential oils and various excerpts from many plants are of outstanding stake in both academia and the food industry, since their potential role as natural additives has emerged from a rising trend to replace synthetic antioxidants with natural ones. In the present work, the MTT assay leads to the formation of formazan due to the presence of madecassoside compound, which was found to be similar with the old reports. The 4-fold dilutions of the aqueous extract incubated with confluent cell monolayers in 96-well plates and were observed microscopically for changes in cell morphology and viability at 24, 48 and 72 hours of incubation (Chiang *et al.*, 2002). Reduced MTT formazan is exocytosed to form needle-like formazan crystals at the cell surface. Mitochondria are unlikely to take on a substantial role in the cellular MTT reduction (Liu, 1999).

The determination is in accordance with the report of Yen and Hsieh (1998), who found that the antioxidative action of leaf extracts of Du-Zhong (*Eucommia ulmoides*) was

more eminent than the raw and roasted cortex of the plant. An important study in *Psidium guajava* significantly puts forward the high content of phenols, flavonols and so forth, which passed on a clear-cut property against free radicals (Ayoola *et al.*, 2008). Most antioxidant activities from plant sources are correlated with phenolic-type compounds (Huang *et al.*, 2010; Sabir and Rocha, 2008). Methanolic extracts from brown seaweed have demonstrated protective effects against both H₂O₂ and tert-BOOH induced reduction in SOD activity (O'Sullivan *et al.*, 2011).

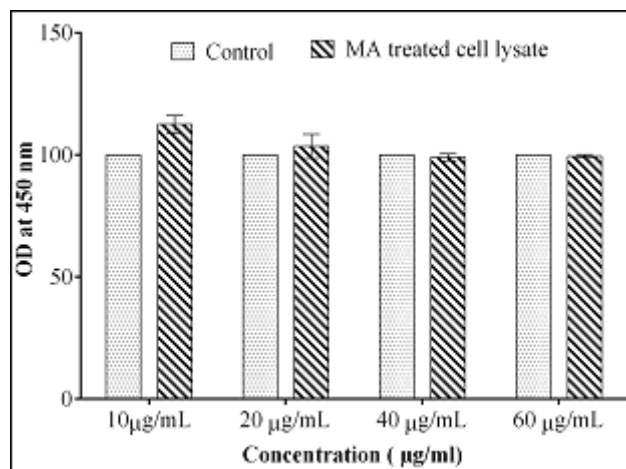


Fig. 5: Effect of madecassoside assayed by the LDH leakage assay. Results are represented as mean $\pm$ standard deviation, $n=3$. Error bars are standard error of mean. Statistical significant differences ($P<0.05$).

The LDH leakage assay and MTT assay the most common employed for the detection of cytotoxicity or cell viability following exposure to toxic substances. The basis for LDH leakage assay is due on the measurement of lactate dehydrogenase activity in the extra cellular medium. The main characteristics invoke reliability, speed and simple evaluation for this assay (Decker and Lohmann-Matthes, 1988). The loss of intracellular LDH and its vent into the culture medium is an indicator of irreversible cell death due to cell membrane damage. The present results also offer evidence that madecassoside contains neuroprotective agents against induced hypoxia. In summation, the traditional utilization of this plant for the treatment of hypoxic-ischemic injury may be ascribed to the presence of prophylactic neuroprotective components. Continued efforts to distinguish the active atoms and will pave the way for further understanding of the neuroprotective nature of madecassoside.

CONCLUSION

The increased concentration of phenolic components justify the effective antioxidant and antitumor activity, majorly the triterpenes namely asiaticoside and madecassoside. In addition, the present work suggest that

the evolution of novel drugs and as a source of antioxidant and neuroprotective pharmaceutical raw material from the plant *C. asiatica*.

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