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Neuroprotection by ethanolic extract of *Syzygium aromaticum* in Alzheimer's disease like pathology via maintaining oxidative balance through SIRT1 pathway

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ABSTRACT

The oxidative stress plays a key role in Alzheimer's disease (AD) and Sirtuin (SIRT1) is potential mediator of oxidative pathway. This study explored the role of *Syzygium aromaticum* on SIRT1 and oxidative balance in amyloid beta induced toxicity. Anti-oxidative capacity of *Syzygium aromaticum* was performed in $A\beta_{25-35}$ induced neurotoxicity in neuronal cells. Superoxide dismutase, Catalase and Glutathione enzyme activity were determined by the treatment of *Syzygium aromaticum*. Both recombinant and endogenous SIRT1 activity were performed in its presence. The expression of γ-secretase and SIRT1 were evaluated by western blot. *Syzygium aromaticum* was capable to scavenge ROS and elevate the percentage of anti-oxidant enzymes. It also activated and elevated the level of SIRT1 and downregulated γ-secretase level. These findings show a holistic approach towards the neurodegenerative disease management by *Syzygium aromaticum* which could lead to the formulation of new drug for AD.

This Ayurvedic product can give a healthy aging with no side effects and also be cost effectives. It may meet unmet medical needs of current relevance.

1. Introduction

Alzheimer's disease (AD) has been recognized 100 years back, yet the exact phenotype of the disease is not known. AD is complex entities due to unfathomgeiable complexities of neural circuits, limited understanding of the organization, functioning of the brain and other components of nervous system. The holistic approach can be better option to prevent and treat the wide-ranging pathological consequences of neurodegenerative disorders due to several unknown factors. Several recent studies, suggest that Ayurveda may indeed provide a good holistic care and treatment in rationale way with different Ayurvedic formulations and their usages (Lakhotia, 2013). Ayurvedic medicines have attracted considerable attention in recent years, as it maintains a perfect healthy aging. Ayurvedic treatment is well known for anti aging. This treatment concentrates on three areas of a person, Vata, Kapha and Pitta and maintaining these three aspects is the secret of healthy aging. In Ayurveda the anti-aging concept is described in Rasayanachikitsa where it describes various herbs and their utility for anti-aging properties (Mehta and Joshi, 2012). Aging is a result of accumulation of cellular damage to proteins and membranes. A possible cause of cellular damage could be accumulation of reactive oxygen species (ROS), notably hydrogen peroxide (H_2O_2) that increases with aging due to the insufficient detoxification, results in oxidative stress (Sohal and Weindruch, 1996). This causes geriatric syndrome and various neurodegenerative diseases. Vata manage movement in the body, the activities of the nervous system and the process of elimination. So variation of vata and vatarakta manifests loss of synaptic transmission, loss of memory and causes various diseases connected to nervous system. Antioxidant ayurvedic product may help in scavenging free radicals generated and prevent from diseases. Calorie restriction (CR) in rodents is associated with deceased level of oxidative damage to DNA. Sirtuin (SIRT) proteins are the mediators of anti-aging effect of CR (Kumar et al., 2014). This protein mediates an oxidative stress response directly by deacetylating several transcription factors that regulate antioxidant genes. SIRT1 increases the activity of α-secretase in neurons and leads to cleavage of amyloid precursor protein (APP) to form non-amyloidogenic peptide. This results in a reduction of toxic amyloidogenic pathway by β-secretase and γ-secretase (Cornier et al., 2017). In our

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previous study, we found serum SIRT1 downregulated in AD patients (Kumar et al., 2013). It can be purposed that the activation of SIRT1 by the Ayurvedic products can avoid ROS formation and protect from neurodegenerative diseases in elderly. *Syzygium aromaticum* (Clove) has highest ORAC (Oxygen Radical Absorbance Capacity) value measured by National Institute on Aging in the National Institutes of Health (NIH) published by United States Department of Agriculture. *Syzygium aromaticum* has a capacity to pacify Vata and thus stimulates nerves (Halder et al., 2011). In this study we explored the role of *Syzygium aromaticum* as anti-oxidant for prevention of AD pathology through activation of SIRT1 pathway in in-vitro model.

2. Materials and methods

2.1. Preparation of ethanolic extract of Syzygium aromaticum

Syzygium aromaticum were crushed in the motor pestle, then soaked properly in ethanol and stirred for 72 h. The supernatant was gently separated and the precipitate was again soaked in the ethanol. This process was repeated until the color of the supernatant becomes light. All the supernatants were pulled and filtered with Whatman filter paper no 1. After filtration the ethanol was evaporated by rotatory evaporator and the remaining fractions were kept under vacuum to remove the smallest fraction of the ethanol present in the extract.

2.2. DPPH radical scavenging activity

Syzygium aromaticum extract (100 µl) were mixed with 100 µl of 0.16 mM DPPH solution. The mixture was vortex, kept for 30 min in dark and the absorbance was measured at 517 nm in an automated microplate reader. The antioxidant capacity was calculated using the following equation: %Inhibition = [control − (sample − blank) / control) × 100], where the control was the absorbance of the DPPH without sample, the sample was the absorbance of the test sample (the sample test and DPPH solution), and the blank was the absorbance of the sample without the DPPH solution. Ascorbic acid was used as positive control.

2.3. Cell cytotoxicity assay on HEK293 cells

SH-SY5Y cells were obtained from NCCS, Pune, India and maintained in Ham's F12 Nutrient media (Gibco) supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic-antimycotic solution (CELL clone, India). The cells used in the experiment were of passage number 31–35. Before starting the experiments, cells were authenticated by STR profiling (DNA forensic laboratory Private limited). The cells were maintained at 37 °C and 5% CO2 under humidified condition. Cells were grown as monolayer.

SH-SY5Y cells were plated at a density of 1×10^6cells per T25 flask. After 24 h of plating, treated with 20 µM retinoic acid in fresh complete media for differentiation (König et al., 1990; Påhlman et al., 1984) for 7 days, where media was changed every 2nd day. On 7th day, cells were treated with 20 µM $A\beta_{25–35}$ synthetic peptide and different concentrations of *Syzygium aromaticum* extract (5, 10 and 50 µg/ml) for 72 h simultaneously. $A\beta_{25–35}$ peptide was synthesized by solid phase peptide synthesis as described in previous paper (Shekhar et al., 2016). The rationale behind using $A\beta_{25–35}$ peptide for toxicity was that, the segment 25–35 of Aβ amyloid42 exhibits physiological and biological properties of Aβ amyloid42 and thus capable of causing neurotoxicity (Ju et al., 2005; Chen et al., 2014; Wu et al., 2016; Kumar et al., 2016).

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay was performed to check the effect of *Syzygium aromaticum* on cell viability of normal cells, HEK293. Cells were maintained in DMEM media with 10% FBS. HEK293 were plated at a density of 5×10^3 cells per well in 96-well plates. After 24 h of plating, cells were treated with different concentrations of *Syzygium aromaticum* for different time intervals i.e. 24 h, 48 h and 72 h. After the completion of treatment time, 10 µl of 5 mg/ml MTT was added to wells and incubated for 4 h at 37 °C. After 72 h media was removed, 100 µl DMSO was added to each well, incubated for 45mins to dissolve the precipitates in the well and absorbance was measured at 572 nm.

2.4. Assessment of rescue effect of Syzygium aromaticum from neurotoxicity of cells

SH-SY5Y cells were plated at a density of 1×10^4cells per well in 96 well plates and the cell viability was determined using the MTT assay. The rescue effect of *Syzygium aromaticum* from $A\beta_{25–35}$ induced neurotoxicity was analyzed by MTT assay as described previously (Shekhar et al., 2018).

2.5. Evaluation the anti-oxidant property of Syzygium aromaticum

2.5.1. Estimation of reduced glutathione (GSH)

The level of reduced glutathione was measured in SH-SY5Y cells treated with *Syzygium aromaticum*. The reduced glutathione assay in cells treated with *Syzygium aromaticum* was done in 96 well plates; the reaction mixture contains 10 µl trichloroacetic acid (10%), 200 µl potassium phosphate buffer (pH 8.0), 0.3 M of 25 µl of 0.04% DTNB and 15 µl of homogenized cell lysates. The absorbance was taken at 412 nm.

2.5.2. Superoxide dismutase (SOD) assay

SH-SY5Y cells were harvested by rubber policeman and collected by centrifuging at 1000–2000 ×*g* for 10 min at 4 °C. Cells were sonicated in cold 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. Then centrifuged at 5000*g* for 10 min at 4 °C, supernatant was collected and stored at −80 °C for further use. The reaction was carried out according to the manufacturer protocol (Cayman superoxide dismutase assay kit 706,002).

2.5.3. Catalase (CAT) assay

Treated cells were harvested in ice cold buffer (50 mM potassium phosphate pH 7.0, 1 mM EDTA) and homogenized by sonication and kept at −80 °C for future use. The reaction was carried out according to the manufactures protocol (Cayman catalase assay kit 707,002).

2.6. Intra-cellular reactive oxygen species detection

ROS production was measured using an oxidation-sensitive fluorescent probe (DCFH-DA). DCFH-DA penetrates cells and gets hydrolyzed by intracellular esterases to the non-fluorescent DCFH, which rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. SH-SY5Y cells were cultured in 96-well plates overnight and then probed with DCFH-DA (100 µM), followed by washing with HBSS (without Ca2+ and Mg2+), then treated with various concentrations (5, 10 and 50 µg/ml) of *Syzygium aromaticum* for 72 h. After incubation period, the reading was observed by spectroflurometer (Molecular Devices, Sunnyvale, California, United States) at excitation of 480 nm and emission of 535 nm.

2.7. Effect of Syzygium aromaticum on the activity of recombinant SIRT1 and SIRT1 in SH-SY5Y cells:

To perform the activity assay, SIRT1 was expressed and purified using bacterial system as mentioned in our earlier paper (Kumar et al., 2012). The extract of *Syzygium aromaticum* was biochemically screened using fluorescence based deacetylase assay to find out its ability to enhance the activity of purified SIRT1. For this assay, 2 and 10 µg/ml of *Syzygium aromaticum* extract were added in two separate well containing 126 ng of SIRT1 Enzyme, 500 µM NAD+ (Enzo Life Sciences), 200 µM SIRT1 peptide substrate (Enzo Life Sciences,) and SIRT1 assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM