

# *In vitro* antioxidant activity of *Lactobacillus plantarum* against hydrogen peroxide-induced neuronal damage on PC12 cells

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## ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease of the central nervous system. Inflammatory injury and oxidative stress have been a hallmark for AD due to free radical production. The main objective of the present study is to identify the compounds having good antioxidant properties from *Lactobacillus plantarum* and its effect on neuronal cells. *L. plantarum* strain was cultured in De Man, Rogosa and Sharpe (MRS) medium supplemented with cinnamon and pantothenic acid (40 µg/ml and 4 µg/ml). Solvent extraction (ethyl acetate, chloroform, petroleum ether, and hexane) was done in the supernatant and tested for *in vitro* 2,2-diphenyl-1-2-picrylhydrazyl (DPPH), H<sub>2</sub>O<sub>2</sub> scavenging activity, and H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity assessment in PC12 cell lines. The results obtained from the study showed that all the extracts have good antioxidant activity. It was observed that the stress-induced PC12 cell lines showed maximum protection of cells in the Media D (chloroform) extract, the IC<sub>50</sub> value was recorded as 23.71 µg/ml. Similarly, all solvent extracts showed significant antioxidant activity in DPPH assay with IC<sub>50</sub> value ranging from 23.59 to 106.8 µg/ml and IC<sub>50</sub> value in H<sub>2</sub>O<sub>2</sub> scavenging assay ranging from 37.39 to 107.7 µg/ml. Referring to the complex multifactorial etiology of AD, the findings from our work exhibited remarkable potentials of antioxidants activity.

## 1. INTRODUCTION

Alzheimer's disease (AD), a significant health complication in the civilized world, is a standard form of dotage-related neurodegenerative dementia. It is a progressive, neurodegenerative disease, the genesis of neurological decline and stands to be an ultimate and eventually a life-threatening disease unless the death is interceded by another cause [1]. AD affects mainly the parts of the brain associated with higher mental functions, specifically the neocortex and hippocampus [2]. AD is mainly characterized by abnormal deposition of extracellular amyloid-beta proteins (Aβ) and intracellular neurofibrillary tangles (tau proteins) and by comprehensive loss of neurons [3]. Etiology of AD is not fully understood because of the multifactorial mechanisms underlying the disease, and various studies suggested that free radicals were involved in the inflammatory injury and oxidative stress in AD [4,3]. The neuronal cell death is caused by the elevated production of reactive oxygen species (ROS) and nitrogen species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitric oxide (NO) generated by Aβ in microglial cells [5,6].

Reactive oxygen species (ROS) play a key role in the pathogenesis of several diseases, including AD [7]. The human brain consumes

~20% of oxygen supply and is more susceptible to oxidative stress due to its high-energy consumption and increased oxidative stress has been reported in AD patients' brain [8]. Oxidative stress is a condition caused by the imbalance between the ROS production and antioxidants levels causing damage to the cells by excessive production of ROS. ROS modulate the function of biomolecules and may target the cell substrates resulting in causing protein, DNA, RNA oxidation, or lipid peroxidation [9].

*Lactobacillus plantarum* MTCC 1325 strain was reported to have the competence of producing acetylcholine (Ach) neurotransmitter through both external and internal pathways and also holds significant antioxidant activity [10,11]. The studies on gut-brain axis communication depict that the bacteria (microbiome) present in the gastrointestinal tract possibly communicate with the brain and nervous system in different ways [12].

Pantothenic acid (Vitamin B5) is a water-soluble vitamin, with the main position for metabolic reactions due to its incorporation into coenzyme A (CoA) and intake of pantothenic acid is reported for the reduction of oxidative stress and improved brain damage caused in gamma-irradiated rats [13,14]. Cinnamon (*Cinnamomum zeylanicum* and *Cinnamomum cassia*), known as the eternal tree of tropical medicine, belongs to the family Lauraceae, besides its antioxidant properties, they are also recorded to have anti-inflammatory, antimicrobial, antidiabetic, anticancer, and lipid-lowering and cardiovascular disease-lowering properties. They are also reported to have activity

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against neurological disorders such as Parkinson's and Alzheimer's disease [15].

The aim of this study is an attempt on the antioxidant capacity of *L. plantarum* grown in MRS culture media supplemented with pantothenic acid and cinnamon as a potential source for the treatment of AD.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism, Fermentation Condition, and Extraction

The strain *L. plantarum* MTCC:1325 obtained from MTCC (Pune, India) was freshly transferred into MRS (De Man, Rogosa and Sharpe) broth (pH 6.5 ± 0.2) at 30°C with agitation 120 rpm for 72 h in an orbital shaker incubator (SLM INC-OS-156). The revived *L. plantarum* was cultured in different types of medium such as Media A, B, C, and D (Media A – MRS medium alone, Media B – MRS + cinnamon (40 µg/mL), Media C – MRS + Pantothenic acid (4 µg/mL), and Media D – MRS + Cinnamon (40 µg/mL) and Pantothenic acid [4 µg/mL]) and incubated for 7–8 days, at 30°C, 120 rpm [16]. The fermented medium was subjected for centrifugation at 8500×g for 5 min at 10°C and the supernatant was filtered using Whatman No.1. The collected filtrates were exhaustively extracted using liquid-liquid extraction using different polarity solvents such as ethyl acetate, chloroform, petroleum ether, hexane, ethanol, and acetone on a separating funnel and the experiment was repeated thrice. The solvent layers of each medium were concentrated under vacuum and transferred to watch glass for further evaporation [17].

### 2.2. 2,2-Diphenyl-1-2-picrylhydrazyl Scavenging Assay

The free radical scavenging activity of the extracts was performed as per DPPH (2,2-diphenyl-1-2-picrylhydrazyl) using Blois [18] method. The DPPH solution was prepared using 0.01 mM in methanol and 1 ml of the solution was transferred into 1 ml of various concentrations (20, 40, 60, 80, and 100 µg/ml) extracts and the OD was taken after 30 min incubation in dark and absorbance measured at 517 nm.

The scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left[ \frac{\text{Absorbance of control} - \text{Absorbance of the sample}}{\text{Absorbance of control}} \right] \times 100$$

The significance of the IC<sub>50</sub> values of standard (ascorbic acid) and the extracts was compared using *t*-test analysis (MS Excel software).

### 2.3. Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging potential of the extracts was determined by H<sub>2</sub>O<sub>2</sub> assay [19], 20 mM H<sub>2</sub>O<sub>2</sub> solution (prepared in phosphate buffer saline [PBS]), and pH (7.4), and various concentrations of extracts (20, 40, 60, 80, and 100 µg/ml) dissolved in 1 ml ethanol were added to the 2 ml H<sub>2</sub>O<sub>2</sub> solution. The absorbance of the samples was measured at 230 nm after 10 min of incubation and the percentage inhibition was calculated. H<sub>2</sub>O<sub>2</sub> solution without solvent extract served as blank and the results (IC<sub>50</sub> values) were compared using *t*-test analysis in MS Excel software to find the significance between standard (ascorbic acid) and extracts.

$$\text{Percentage Scavenged [H}_2\text{O}_2\text{]} = \left[ \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right] \times 100$$

### 2.4. Cell Culture and Treatment

Rat pheochromocytoma (PC12) cells were obtained from ATCC (Pune, India) and were grown in Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 5% fetal bovine serum (FBS), 10% heat-inactivated horse serum, and 1% pen-strep (penicillin-streptomycin) and incubated at 37°C in 95% air and 5% CO<sub>2</sub> humidified incubator [20]. A density of 100,000 cells/ml was seeded in 96-well plates (100 µl of cells per well) and the cells were pre-treated with various concentration of extracts (20, 40, 60, 80, and 100 µg/ml) followed by the addition of H<sub>2</sub>O<sub>2</sub> (500 µM) (for the induction of neuronal damage) and the cells were incubated for 24 h and galantamine (USFDA approved drug for AD) was used as positive control and cells exposed with H<sub>2</sub>O<sub>2</sub> were used as a negative control [3].

### 2.5. Cytotoxicity Assessment

Cytotoxicity of cells was determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT dye). Cells pre-treated and incubated with extracts and H<sub>2</sub>O<sub>2</sub> for 24 h were then incubated with MTT dye (5 mg/ml) for 4 h, and after incubation, the supernatant was removed and added with 150 µl of dimethyl sulfoxide. The plates were then kept in a shaker for 10 min and absorbance was taken at 535 nm on a microplate reader (BIO-TEK 800 TS) [3,20]. The experiment was carried out in triplicates and the MTT reduction was estimated as a percentage of the control cell absorption and IC<sub>50</sub> were calculated (optical density of the cell viability of cells without any treatment was 100%).

## 3. RESULTS AND DISCUSSION

*L. plantarum* MTCC NO:1325 grown in Media A, B, C, and D were extracted using liquid-liquid extraction with ethyl acetate, chloroform, petroleum ether, hexane, acetone, and ethanol. Acetone and ethanol were completely got miscible with culture medium and were omitted for further extraction procedures. The extracts were then quantitatively evaluated for antioxidant activity.

DPPH is a stable free radical widely used for the investigation of the scavenging activity of compounds, this stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The antioxidant turns the dark blue (deep violet color) color of DPPH radical solution and makes it lighter (yellow) colored α, α-diphenyl-β-picryl hydrazine, thus the discoloration indicates the radical scavenging potential activity of the antioxidant compound or the extracts used in the term of hydrogen or electron-donating ability. This assay is very useful to find a stable antioxidant drug that can reduce the generated free radicals [19,21]. All 16 extracts were subjected to DPPH scavenging activity and showed activity in a dose-dependent manner [Table 1-4]. The percentage inhibition of DPPH scavenging activity was calculated for each concentration of individual extracts. Ascorbic acid was used as a positive control. The concentration of the extract required to bring about 50% of the original activity (IC<sub>50</sub> value) was determined for each extract. The extracts could neutralize the DPPH free radicals through hydrogen-donating activity. Tables 1-4 were compared and the highest activity was detected in Media C (chloroform [Table 2]) (IC<sub>50</sub> value 23.59 µg/ml), followed by Media D (chloroform [Table 2]), Media B (hexane [Table 4]), and Media B (ethyl acetate [Table 1]) (IC<sub>50</sub> value 31.65, 35.66, and 39.92 µg/ml, respectively). The *t*-test (MS Excel software) comparison on ethyl acetate [Table 1], chloroform [Table 2], petroleum ether [Table 3], and hexane extracts [Table 4] with the standard shows that there is a significant difference between the extracts and ascorbic acid with an IC<sub>50</sub> value of 29.85 µg/ml.

H<sub>2</sub>O<sub>2</sub> is a weak oxidizing agent and by oxidation of essential thiol (–SH) groups, they are capable of inactivating a few enzymes directly

**Table 1:** IC<sub>50</sub> values of *in-vitro* antioxidant assay and H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay of ethyl acetate extracts

Media	IC <sub>50</sub> value DPPH assay±SEM (µg/ml)	IC <sub>50</sub> value H <sub>2</sub> O <sub>2</sub> assay±SEM (µg/ml)	IC50 value H <sub>2</sub> O <sub>2</sub> induced cytotoxicity assay±SEM (µg/ml)
Media A (Ethyl acetate)	91.12±11.6	52.4±8.53	73.35±0.85
Media B (Ethyl acetate)	39.92±6.51	83.5±8.6	51.01±1.77
Media C (Ethyl acetate)	40.56±7.83	53.71±11.62	80.98±1.94
Media D (Ethyl acetate)	42.07±9.15	67.37±3.76	84.9±2

Values are expressed as mean±SD\* (n=3). Ascorbic acid was used as a control for antioxidant assays. IC<sub>50</sub> for the positive control for DPPH scavenging assay was 29.85±9.14 µg/ml, Hydrogen peroxide scavenging assay was 25.69±3.42 µg/ml, IC<sub>50</sub> for galantamine positive control for H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay was 19.29±0.85

**Table 2:** IC<sub>50</sub> values of *in-vitro* antioxidant assay and H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay of chloroform extracts

Media	IC <sub>50</sub> value DPPH assay±SEM (µg/ml)	IC <sub>50</sub> value H <sub>2</sub> O <sub>2</sub> assay±SEM (µg/ml)	IC50 value H <sub>2</sub> O <sub>2</sub> induced cytotoxicity assay±SEM (µg/ml)
Media A (Chloroform)	88.45±11.17	86.46±4.24	39.48±1.09
Media B (Chloroform)	41.84±8.17	56.15±8.93	64.09±1.86
Media C (Chloroform)	23.59±7.83	42.17±8.73	89.88±2.07
Media D (Chloroform)	31.65±9.15	38.17±4.19	23.71±1.40

Values are expressed as mean±SD\* (n=3). Ascorbic acid was used as a control for antioxidant assays. IC<sub>50</sub> for the positive control for DPPH scavenging assay was 29.85±9.14 µg/ml, Hydrogen peroxide scavenging assay was 25.69±3.42 µg/ml, IC<sub>50</sub> for galantamine positive control for H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay was 19.29±0.85

**Table 3:** IC<sub>50</sub> values of *in-vitro* antioxidant assay and H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay of petroleum ether extracts

Media	IC <sub>50</sub> value DPPH assay±SEM (µg/ml)	IC <sub>50</sub> value H <sub>2</sub> O <sub>2</sub> assay±SEM (µg/ml)	IC50 value H <sub>2</sub> O <sub>2</sub> induced cytotoxicity assay±SEM (µg/ml)
Media A (Petroleum ether)	104±12.32	62.17±9.85	60.43±1.9
Media B (Petroleum ether)	42.37±9.1	95.63±20.12	92.6±2.02
Media C (Petroleum ether)	40.62±9.02	37.39±4.52	64.45±1.88
Media D (Petroleum ether)	52.28±8.23	67.06±14.01	82.87±1.97

Values are expressed as mean±SD\* (n=3). Ascorbic acid was used as a control for antioxidant assays. IC<sub>50</sub> for the positive control for DPPH scavenging assay was 29.85±9.14 µg/ml, Hydrogen peroxide scavenging assay was 25.69±3.42 µg/ml, IC<sub>50</sub> for galantamine positive control for H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay was 19.29 ± 0.85

and are rapidly able to cross cell membranes and inside cells. The toxic effects of H<sub>2</sub>O<sub>2</sub> originate from the most reactive free radical called hydroxyl radical which is possibly be formed from a superoxide anion and H<sub>2</sub>O<sub>2</sub> in the presence of metal ions such as Fe<sup>2+</sup> and Cu<sup>2+</sup> [22]. The accumulation of H<sub>2</sub>O<sub>2</sub> was reported to cause various conditions such as cancer, ischemia, and neurodegenerative disease [23]. The

**Table 4:** IC<sub>50</sub> values of *in-vitro* antioxidant assay and H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay of hexane extracts

Media	IC <sub>50</sub> value DPPH assay±SEM (µg/ml)	IC <sub>50</sub> value H <sub>2</sub> O <sub>2</sub> assay±SEM (µg/ml)	IC50 value H <sub>2</sub> O <sub>2</sub> induced cytotoxicity assay±SEM (µg/ml)
Media A (Hexane)	106.8±13.1	52.95±5.68	41.81±1.66
Media B (Hexane)	35.66±9.01	107.7±8	69.74±1.86
Media C (Hexane)	70.58±17.43	41.19±5.99	72.92±1.92
Media D (Hexane)	40.28±6.77	51.14±7.93	32.82±1.56

Values are expressed as mean±SD\* (n=3). Ascorbic acid was used as a control for antioxidant assays. IC<sub>50</sub> for the positive control for DPPH scavenging assay was 29.85±9.14 µg/ml, Hydrogen peroxide scavenging assay was 25.69±3.42 µg/ml, IC<sub>50</sub> for galantamine positive control for H<sub>2</sub>O<sub>2</sub> induced cytotoxicity assay was 19.29±0.85

accumulation control of the amount of H<sub>2</sub>O<sub>2</sub> is, therefore, biologically an advantage for cells. Ascorbic acid was used as positive control. The highest activity was detected in Media C (petroleum ether) (IC<sub>50</sub> value 37.39 µg/ml [Table 3]), followed by Media D (chloroform [Table 2]), Media C (hexane [Table 4]), and Media C (chloroform [Table 2]) (38.17, 41.19, and 42.17 µg/ml, respectively). The *t*-test (MS Excel software) done in the H<sub>2</sub>O<sub>2</sub> scavenging assay shows that there is a significant difference between the extracts and ascorbic acid with IC<sub>50</sub> value 25.69 µg/ml [Table 1-4].

Studies were done by Ezoulin *et al.* [3] in neuronal damage induced by H<sub>2</sub>O<sub>2</sub> in SK-N-SH cells confirm that accumulation of ROS and nitrogen species is associated with loss of mitochondrial activity which is characterized by the cell damage and oxidative stress caused by H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide has been used extensively as apoptosis and necrosis induced in various cell types [24]. Prevention of apoptosis induced by Aβ in human neuroblastoma cell line SH-SY5Y by galantamine was previously reported in the studies done by Arias *et al.* [25]. Our study in H<sub>2</sub>O<sub>2</sub>-induced neuronal damage in a PC12 cell line with extracts and galantamine (positive control) showed activity in a dose-dependent manner. From the results obtained [Table 1-4], the extracts can serve as a good antioxidant source for neuronal damage caused by H<sub>2</sub>O<sub>2</sub>. Maximum protection of cells was observed in Media D (chloroform [Table 2]) (IC<sub>50</sub> value 23.71 µg/ml), followed by Media D (hexane [Table 4]), Media A (chloroform [Table 2]), and Media A (hexane [Table 4]) (IC<sub>50</sub> value 32.82, 39.48, and 41.81 µg/ml, respectively) and positive control galantamine with IC<sub>50</sub> value 19.29 µg/ml.

#### 4. CONCLUSION

To the best of our knowledge, this study has been the first report on antioxidant activity and H<sub>2</sub>O<sub>2</sub> neuronal damage induction study in *L. plantarum* MTCC NO 1325 grown in media containing cinnamon and pantothenic acid. Results attained from this study indicated that *L. plantarum* MTCC NO 1325 extracts had considerable antioxidant activity against various antioxidant systems *in vitro*. *L. plantarum* grown in MRS medium extracts obtained results show a low or no activity but the same microbe was grown in cinnamon and pantothenic acid showed a very high antioxidant activity. Thus, the expression of the secondary metabolites produced by the microbe was manipulated by the alteration of the fermentation media. Inflammatory injury and oxidative stress have been a hallmark for AD due to free radical production. Thus, depletion of reactive oxygen species like H<sub>2</sub>O<sub>2</sub> can reduce neuronal cell damage. From the results stated above, it

can be concluded that the preponderance of antioxidant activity of *L. plantarum* MTCC-1325 in cinnamon and pantothenic acid was proved to be correct, and in future, it can be used as a natural source of antioxidant with consequent health benefits.

## 5. CONFLICT OF INTEREST

Authors declared that there are no conflicts of interest.

## 6. FINANCIAL SUPPORT AND SPONSORSHIP

None.

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