

Dose-dependent Effects of Fermented *Mucuna pruriens* Extract on Dopaminergic Neuron Viability *in vitro*

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Abstract. *Mucuna pruriens* are traditionally used as the main ingredient for fermented food called *Tempe Koro Benguk*. In addition to their value as food commodities, *M. pruriens* seeds are rich in levodopa (L-DOPA), a precursor to dopamine that holds potential therapeutic benefits for several neurodegenerative disorders such as Parkinson's disease. This study investigated the effects of ethanolic and propanolic extracts derived from fermented *Tempe Koro Benguk* on the viability of dopaminergic neurons cultured from fetal rat ventral midbrains. *Tempe Koro Benguk* extracts were obtained using ethanol and n-propanol as solvents and were administered at varying concentrations (0.05 μ M, 0.5 μ M, 5 μ M and 50 μ M) to primary midbrain cultures harvested on gestational period day 14. After 10 days of exposure, the cells were fixed and immunohistochemically stained for tyrosine hydroxylase (TH) to visualize the dopaminergic neurons. Our study demonstrated that the highest viability of TH-immunoreactive cells was observed in cultures treated with ethanolic extract at 0.05 μ M concentration. Although not statistically significant, this number was slightly higher than that of the control levels. However, both ethanolic and propanolic extracts at concentrations of 5 μ M and 50 μ M were associated with a complete loss of TH-immunoreactive neurons. Notably, although the ethanolic extract at 0.5 μ M yielded well defined morphological features of dopaminergic neurons, viability remained below control levels. These findings suggest that *Tempe Koro Benguk* extract may offer dose-dependent neuroprotective properties, with low concentrations potentially supporting dopaminergic neurons survival. Further studies are required to elucidate the mechanisms involved and to evaluate its safety and efficacy profiles.

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1 INTRODUCTION

Mucuna pruriens, a tropical legume, is widely recognized by several names, including velvet bean, cowitch, and Kawaanch [1]. It is among the most prominent medicinal plants in India, forming the basis of over 200 traditional drug formulations [2]. Every part of *M. pruriens* holds medicinal value. Its roots, leaves, and seeds are commonly used to treat male infertility, snake bites, diabetes, cancer, and several neurological disorders, including Parkinson's disease (PD) and Alzheimer's disease [3]. In Indonesia, various beans, including *M. pruriens* bean, have been fermented to produce *Tempe*, a traditional product originally developed by the Central Javanese locals [4]. *M. pruriens* seeds, commonly known in Indonesia as *Koro Benguk*, are the main ingredient in a traditional fermented food called *Tempe Koro Benguk*, which is well known for its distinctive flavour and cultural significance. According to previous study, the extract of *Tempe Koro Benguk* contains levodopa (L-DOPA), albeit in very low concentrations [5]. Despite being widely consumed as traditional fermented food, the benefits of *Tempe Koro Benguk* on dopaminergic neurons and its potential role as an alternative treatment for PD remain unexplored.

The use of *M. pruriens* seed powder to treat Parkinson's symptoms in humans and animal models has been extensively studied [6]. This is because the seeds of *M. pruriens* from various countries contain high concentrations of L-DOPA [1,6]. Since its introduction in the late 1960s, L-DOPA remains the most effective and gold-standard therapy for PD [7]. A previous study has explored the neuroprotective mechanisms of *M. pruriens* seeds extracts on dopaminergic neurons in animal models of neurodegenerative diseases [8]. However, no research has explored the effects of extracts derived from fermented *M. pruriens* seeds, or *Tempe Koro Benguk*, on primary dopaminergic neuron cultures.

This study aimed to evaluate the effect of *Tempe Koro Benguk* fermentation extract supplementation on dopaminergic neurons cultured from fetal rat ventral midbrain at embryonic day 14. The findings provide insights into the potential benefit of consuming *Tempe Koro Benguk* on dopaminergic neurons and offer foundational data for future research exploring its potential role as an alternative treatment for PD.

2 Material and methods

2.1 Ethical approval

This study was conducted in full accordance with ethical standards for animal research. The protocol used in this study was approved by the ethics committee with reference number of 289/KEC-LPPT/VI/2015, ensuring compliance with guidelines for animal welfare and research ethics.

2.2 Preparation of '*Tempe Koro Benguk*' extracts

M. pruriens seeds were obtained from Special Region of Yogyakarta, Indonesia and its surrounding areas. The seeds were fermented to produce *Tempe Koro Benguk*. The extraction process was carried out at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada. The extractions were performed following the previously described procedure [9]; using two different solvents: first extraction was carried out using ethanol (ethanolic extraction) and the other using n-propanol (propanolic extraction).

2.3 Dopaminergic Neurons Cell Cultures

Fetal rat brains were obtained from pregnant female rats at gestational day 14. The fetuses were prepared, and the brains were collected. The brains were placed in cold phosphate-buffered saline (PBS) solutions (4°C) in sterile Petri dishes. The ventral midbrain, of mesencephalon, was dissected and subsequently transferred to a 15 mL conical tube containing 0.5 mL of Minimum Essential Medium (MEM) supplemented with 10% horse serum and 10% Fetal Bovine Serum (FBS). The sample was then centrifuged at 200g for 5 minutes. The resulting pellet was manually dissociated into a single-cell suspension using a micropipette, followed by the addition of 4 mL of medium.

The mixture was centrifuged again at 200g for 5 minutes at 4°C. After removing the supernatant, 3 mL of media was added, and the pellet was dissociated once more. The resulting cells were seeded onto collagen-coated flasks and incubated in a CO₂ incubator set at 5% CO₂ and 37°C for 1 hour. Following incubation, 3 mL of MEM was added to each flask, and the culture was further incubated for an additional 3 days. On the fourth day of incubation, the cells were transferred into nine collagen-coated wells. On the fifth day, the medium in each well was refreshed with MEM supplemented with 5% horse serum. From the sixth to the ninth day, the medium was changed daily with MEM supplemented with 5% horse serum. Ethanolic and propanolic extracts of *Tempe Koro Benguk* were added to wells 1, 2, 4, 5, 6, 7, and 8 at concentration of 0.05 µM, 0.5 µM, 5 µM, and 50 µM, respectively. Well 9, serving as the control, did not receive any extract. On the tenth day, the cells in all wells were fixed with 4% paraformaldehyde in PBS for 30 minutes to prepare for immunohistochemical staining.

2.4 Immunohistochemistry staining

Dopaminergic neurons in cell cultures were identified using immunohistochemical staining with the rabbit anti-tyrosine hydroxylase primary antibody (TH) (1:500, Boster Biological Technology, Cat. No: PB9449, California, USA) and the Starr Trek Universal HRP Detection Kit (Biocare Medical, Cat. No: STUHRP700 H, L10, USA). After fixation, all wells were washed with 1% Bovine Serum Albumin (BSA) in PBS. Endogenous peroxidase activity was inhibited by treating the wells with H₂O₂ for 10 minutes, followed by another wash with 1% BSA in PBS. To minimize nonspecific binding, the cultures were incubated with Background Sniper. Without an intermediate washing step, the primary anti-tyrosine hydroxylase antibody (1:1000) was applied, and the cultures were incubated overnight at 4°C. The next day, Trekkie Universal Link was applied for 20 minutes at room temperature. After washing, TrekAvidin-HRP was added and incubated for 10 minutes at room temperature. Antibody binding was visualized using DAB⁺ substrate, followed by counterstaining with Harris Hematoxyline.

2.5 statistical analysis

Dopaminergic neurons in the cell cultures were analyzed both descriptively and quantitatively. The morphology of dopaminergic neurons was observed using an inverted microscope. Immunoreactive cells were counted from five fields of view per well at 100x magnification. Initially, the MEANS procedure was performed to determine the mean and standard deviation immunoreactive cells from each treatment. The obtained descriptive statistical data of each group were then statistically compared using one way ANOVA. A *P*-value <0.05 was considered statistically significant and followed by *post hoc* testing. All statistical analysis was performed using SPSS software, version 16.0.

3 Results

3.1 anti-Tyrosine Hydroxylase-Immunoreactive Cells

Observation using an inverted microscope revealed several anti-tyrosine hydroxylases (TH)-immunoreactive cells in the immunohistochemically stained cell cultures. These TH-immunoreactive cells appeared as dark brown and were identified as dopaminergic neurons. In cell cultures, dopaminergic neurons were organized into grape-like clusters, each containing multiple neurons. The neurons were interconnected, with their cytoplasmic processes linking clusters. Additionally, individual neurons were often observed around the clusters. Dopaminergic neurons exhibited a variety of shapes; while most were bipolar, unipolar and multipolar neurons were also frequently seen (Figure 1).

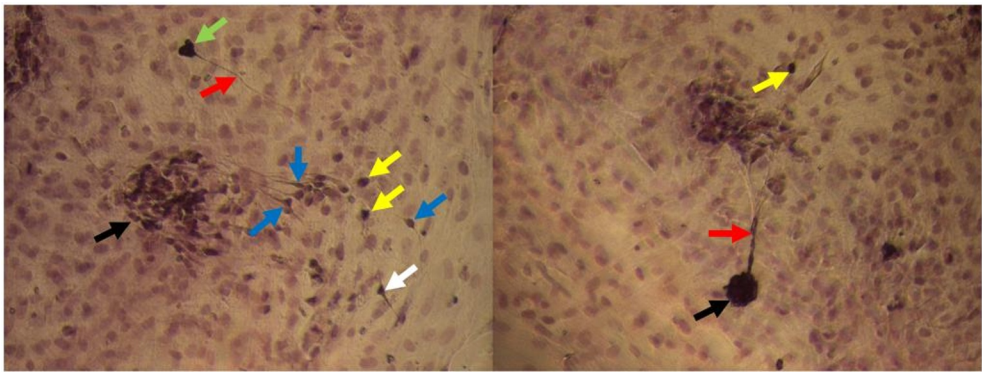


Fig. 1. anti-Tyrosine Hydroxylase-immunoreactive cells identified as dopaminergic neurons, appearing as dark brown. Black arrow: grape-like clusters of dopaminergic neurons, blue arrow: bipolar neuron, green arrow: unipolar neuron, and white arrow: multipolar neuron.

Cell cultures of dopaminergic neurons exhibited different viability in response to varying concentrations of ethanolic and propanolic extracts of *Tempe Koro Benguk*. The highest number of TH-immunoreactive cells was observed in cultures treated with 0.05 μM ethanolic extract, averaging 130.2 ± 7.3 cells per field of view. This number, although not significantly significant ($P = 0.79$), was higher than that of the control wells, which had an average of 92.2 ± 10.3 cells per field of view. In contrast, higher concentrations of ethanolic extract resulted in the reduction of dopaminergic neuron viability. At 0.5 μM , the total number of TH-immunoreactive cells declined, with an average of 75.8 ± 6.9 cells per field. At 5 μM and 50 μM concentrations, no TH-immunoreactive cells were observed. Different results were obtained in cultures treated with various concentrations of the propanolic extract of *Tempe Koro Benguk*. Viable dopaminergic neurons were observed only at a 0.05 μM concentration of the propanolic extract, with an average of 96 ± 24.8 cells per field, while no viable dopaminergic neurons were observed in wells treated with other concentrations (Table 1).

Table 1. Number of anti-tyrosine hydroxylase-immunoreactive cells in cell cultures treated with varied concentrations of ethanolic and n-propanolic extracts of *Tempe Koro Benguk*.

Cell treatment	Number of immunoreactive cells in each field of view					Mean ± SEM
	1	2	3	4	5	
Ethanolic extract 0,05 µM	141	116	114	128	152	130.2 ± 7.3
Ethanolic extract 0,5 µM	71	67	73	103	65	75.8 ± 6.9
Ethanolic extract 5 µM	0	0	0	0	0	NA
Ethanolic extract 50 µM	0	0	0	0	0	NA
Propanolic extract 0,05 µM	42	178	53	85	122	96.0 ± 24.8
Propanolic extract 0,5 µM	0	0	0	0	0	NA
Propanolic extract 5 µM	0	0	0	0	0	NA
Propanolic extract 50 µM	0	0	0	0	0	NA
Control	125	101	63	91	81	92.2 ± 10.3

NA: Not assessed.

3.2 Morphology of Dopamine Neurons

The morphological characteristics of TH-immunoreactive cells varied across wells. The most well-defined morphology among the treatments was observed from dopaminergic neurons treated with 0.5 µM ethanolic extract of *Tempe Koro Benguk*, although the overall cell morphology was still inferior to the controls. Dopaminergic neurons treated with 0.5 µM ethanolic extract of *Tempe Koro Benguk* exhibited clustered cell somata and cytoplasmic processes; however, these features were less well defined compared to that of the control cells, which displayed round grape-like somata and distinct cytoplasmic processes. The dopaminergic neurons from wells treated with 0.05 µM propanolic extract of *Tempe Koro Benguk* showed the least defined cell soma and less cytoplasmic processes (Figure 2).

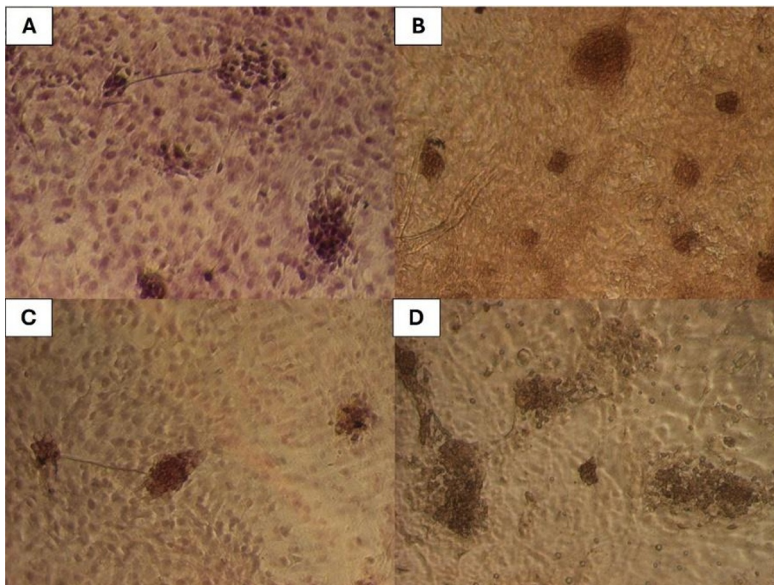


Fig. 2. Morphology of dopaminergic neurons under different treatment conditions. A: control, B: ethanolic extract 0.05 µM, C: ethanolic extract 0.5 µM, D: propanolic extract 0.05 µM.

4 Discussions

Dopaminergic neurons produce dopamine, an essential neurotransmitter in the brain that plays a significant role in maintaining central nervous system (CNS) function. Most dopaminergic neurons are found in the ventral region of the mesencephalon, or midbrain, where they primarily release dopamine, a crucial neurotransmitter in the brain [10]. Dopamine deficiency is linked to motor control disorders such as rigidity, akinesia, tremors, and postural abnormalities. The loss of dopaminergic neurons also leads to several conditions including Parkinson's disease (PD) that remains as a global health burden that associated with the non-communicable diseases [11].

The current gold standard for PD therapy is levodopa (L-DOPA), a dopamine precursor that possesses the ability to replace the pathological loss of dopamine. Although L-DOPA has been a life-changing therapy, several challenges remain, including limited accessibility and high cost. In contrast, *M. pruriens*, a tropical leguminous plant, has shown therapeutic potential for PD due to its naturally high L-DOPA content [5]. In Indonesia, *M. pruriens* beans are commonly fermented to produce one of the most common food commodities called *Tempe Koro Bengkulu*. Although various studies have demonstrated the therapeutic potential of *M. pruriens* beans, the effect of the fermented form remained to be elucidated.

In this study, we evaluated the effects of ethanolic and propanolic extracts of fermented *M. pruriens* seeds on dopaminergic neurons cultured from fetal rat ventral midbrain. Rat embryos are preferred due to the easier dissection process. These primary cultures are widely used to evaluate neuroprotective agents and investigate the properties of dopaminergic neurons [12]. Our findings from primary cultures of dopaminergic neurons derived from fetal rats at gestation day 14 are consistent with those reported in studies on primary mesencephalic neuron cultures from embryonic mice [12]. The morphology of dopaminergic neurons in our cultures closely resembled that observed in embryonic mouse cultures. Neuronal survival in culture can be enhanced by the addition of serum to the culture medium [13]. Immunohistochemical staining with tyrosine hydroxylase (TH) antibodies is a reliable method to confirm the morphology of dopaminergic neurons.

In this study, ethanol and propanol solvents were employed to extract the fermented *M. pruriens* beans due to their superiority to allow highly efficient extraction procedures for variety compounds compared to other solvents such as water and acetone. However, the addition of different compounds to the culture medium can influence cell viability both positively and negatively. Our findings suggest that both ethanolic and propanolic extracts of fermented *M. pruriens* seeds showed highest TH-immunoreactive cells at the lowest concentration, which is 0.05 μ M. The number of viable dopaminergic neurons was slightly higher in ethanolic extraction compared to those of propanolic extraction and the controls, although it was not statistically significant. This finding was in line with previous studies that demonstrated higher L-DOPA contains from ethanolic extraction, that in consequence, might offer higher neuroprotective property [5]. At higher concentrations, both ethanolic and propanolic extracts negatively impacted dopaminergic neuron viability. These findings suggested that the fermented *M. pruriens* seeds might support neuronal survival, however, higher dose and/or different solvents can be toxic.

Moreover, ethanolic extract from fresh *M. pruriens* (L.) seeds exhibit strong antioxidant activity, including the inhibition of 2,2-Diphenyl-1-picrylhydrazyl, hydroxyl radicals, nitric oxide, and superoxide anions, as well as enhanced reducing power in *in vitro* assays [14]. Moreover, extracts from the whole *M. pruriens* plants are rich in phenolic compounds, which have demonstrated significant free radical scavenging and antioxidant properties [15].

5 CONCLUSION

In conclusion, the ethanolic extract of *Tempe Koro Benguk* from fermented *M. pruriens* seeds demonstrated a positive effect on the viability of dopaminergic neurons, with the highest number of immunoreactive cells observed at 0.05 μM concentration, although this number was not significantly different with those of propanolic extraction and control. Higher concentrations of both ethanolic and propanolic extracts negatively impacted dopaminergic neuron viability, with complete inhibition of cell growth at concentrations of 5 μM and 50 μM . These findings suggest that *Tempe Koro Benguk* extract may have potential neuroprotective effects at low concentrations, however, further research is required to evaluate its effectiveness and safety, particularly for potential use in Parkinson's disease treatment.

ABBREVIATIONS

TH, anti-tyrosine hydroxylase; L-DOPA, levodopa; μM , micromolar; $^{\circ}\text{C}$, degrees Celsius, mL, millilitre; g, gram; CO_2 , carbon dioxide; H_2O_2 , hydrogen peroxide; DAB, 3,3'-Diaminobenzidine; SPSS, statistical Package for the Social Sciences.

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TWP conceptualized and designed the experiments while overseeing the research. TWP, SWW, ER, and YKA carried out laboratory experiments and bioinformatics analysis. SWW and YKA drafted the original manuscript, which TWP reviewed and revised. All authors contributed to the final manuscript's revision and approval.

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