

In-vitro Screening of Anti Parkinson's Activity of Ethanolic Extract of *Malaxis acuminata* against Rotenone – Induced Toxicity in Sh-Sy5y Neuroblastoma Cell Lines

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ABSTRACT

The study attempts to investigate *in-vitro* screening of anti-Parkinson's activity of the entire plant of *Malaxis acuminata* by using the SH-SY5Y cell line. The powdered plant of *Malaxis acuminata* was extracted by the Soxhlet method using nonpolar to polar solvents. Rotenone (10µM) was used to induce toxicity on SH-SY5Y cell lines. The cell lines were then treated with 100µg, 50µg, 25µg, 12.5µg, 6.25µg of ethanolic extract of *Malaxis acuminata* and in a humidified 5 % CO₂ incubator, the cells were inoculated at 37°C. When the doses were steadily increased over 24 hr, the percentage viability of the cells fell. 6.25g/ml was shown to be the most effective concentration for inhibiting cell proliferation. The number of viable cells tends to decrease as that the concentration of the extract increases. *Malaxis acuminata* ethanolic extracts exhibited antiparkinsonian activity on SH-SY5Y cell lines.

Key words: Anti-parkinsonian activity, *Malaxis acuminata*, Rotenone, SH -SY5Y cell lines, Jeevak, MTT assay.

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INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative condition typified by the apoptotic cell death in dopamine neurons in the pars compacta, a portion of the substantia nigra located in the midbrain (SNpc), and the outspread of the intracellular protein alpha-synuclein (αSyn). Classic Parkinsonian motor syndrome as bradykinesia, tremor, rigidity, and eventually postural instability, are caused by dopamine deprivation in the basal nuclei. Non-motor symptoms are also linked to

Parkinson's disease, and they can appear more than a decade before motor symptoms. Many of the motor and non-motor symptoms of Parkinson's disease are caused by a neurodegenerative condition in which the brain chemical dopamine is lost, notably anxiety, akathisia, depression, dysautonomia, insomnia, and other sleep problems. The recognition that replenishment of brain dopamine with levodopa brings a Parkinson's patient to life brings. When caring for patients with Parkinson's disease who are hospitalized or admitted to nursing homes, having a basic understanding of levodopa dose and dynamics is essential.^[1,2] Parkinson's disease is caused by the gradual degradation of dopamine-producing nerve cells in the brain. Dopamine is a natural molecule produced in the brain that plays an important part in our brains and bodies by interacting and messaging across multiple systems. Pesticides, herbicides, and proximity to industrial operations are all factors to

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consider. A toxic substance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) builds up in the mitochondria induces Parkinson's disease. The thalamic nuclei are damaged due to oxidation and the formation of free radicals. If one family member has Parkinson's disease, the chance of PD in siblings is enhanced. The dysfunction of alpha-synuclein potentially contributes to the progression of Parkinson's disease. The current study is aimed at preventing alpha synuclein from spreading and aggregating. The major pathogenic feature of PD is cell loss in the basal ganglia of the brain. In Parkinson's disease, alpha-synuclein becomes dysregulated and clumps together with other alpha-synuclein.^[3]

The major pathogenic feature of PD is cell loss in the basal ganglia of the brain. Because cells can't get rid of these clumps, alpha-synuclein becomes cytotoxic, causing cell death. Lewy bodies are aggregates that can be detected in neurons under a microscope. The loss of neurons is accompanied by the death of astrocytes (star-shaped glial cells) and a significant increase in the number of microglia (another kind of glial cell) in the substantia nigra. Braak staging is a method of describing how the areas of the brain affected by Parkinson's disease advance. There are five primary routes in the brain that connect the basal ganglia to other parts of the brain. The motor, oculomotor, associative, limbic, and orbitofrontal circuits are the designations given to each circuit's major projection area. All of them are impaired in Parkinson's disease, and their disruption explains many of the disease's symptoms, as these circuits are engaged in a wide range of processes, such as movement, attention, and learning.^[4] Even though there is no cure for Parkinson's disease, medications, surgical treatment, and other therapies can help to alleviate some of the symptoms. Medications that raise dopamine levels in the brain, drugs that influence other brain chemicals in the body, and drugs that help regulate non-motor symptoms are all recommended for Parkinson's disease. Levodopa, also known as L-dopa, is the most common Parkinson's treatment. To restore the brain's diminishing supply of dopamine, nerve cells employ levodopa to create it. Levodopa is usually combined with another medicine called carbidopa. Carbidopa reduces the quantity of levodopa required to treat symptoms and prevents or reduces some of the side effects of levodopa therapy, such as nausea, vomiting, low blood pressure, and restlessness. Parkinson's patients should never discontinue taking levodopa without first consulting their physician. Stopping the medicine suddenly might have major consequences, such as being unable to move or having trouble breathing.^[5]

DRUG CLASSIFICATION^[6-9]

MAO B inhibitors

These medications include Selegiline (Zelapar), Rasagiline (Azilect), and Safinamide (Xadago). They help prevent the breakdown of brain dopamine by inhibiting the brain enzyme Monoamine oxidase B (MAO B). This enzyme metabolizes brain dopamine. Selegiline given with Levodopa may help prevent wearing-off. Side effects of MAO B inhibitors may include headaches, nausea, or insomnia. When added to Carbidopa-levodopa, these medications increase the risk of hallucinations. These medications are not often used in combination with most antidepressants or certain narcotics due to potentially serious but rare reactions. Check with your doctor before taking any additional medications with an MAO B inhibitor.

Dopamine agonists

Dopamine receptor agonists came into the market for the treatment of PD in 1978. The commonly used agonists contain an ethanolamine moiety, and they may be categorized into ergot and non-ergot derived, based on receptor specificities. These drugs stimulate the activity of the dopamine system by binding to the dopaminergic receptors and, unlike levodopa, do not need to be converted into dopamine. Dopamine agonists are often prescribed as initial therapy for PD, particularly in younger patients. This approach allows for a delay in the use of levodopa, which may reduce the impact of the problematic motor complications, discussed above. Some of the drugs are no longer used in clinical practice, as significant idiosyncratic adverse effects were observed. For example, pergolide was withdrawn as a treatment in 2007, after studies found that it was associated with a risk of pericardial, retroperitoneal, and pleural fibrosis.

Anticholinergics

The medications that have so far been discussed are all designed to increase dopaminergic activity in the striatum. There are a small number of drugs used in the treatment of PD that act through non-dopaminergic mechanisms. One such class of drugs is anticholinergics. These reduce the activity of the neurotransmitter acetylcholine, by acting as antagonists at cholinergic receptors. While their role is limited and they are now prescribed infrequently, they may offer some benefit in improving rigidity and tremor in PD. Loss of dopaminergic neurons results in the disturbance of the normal balance between dopamine and acetylcholine in the brain, and anticholinergic drugs may lead to restoration and maintenance of the normal balance

between these two neurotransmitters. The main role of these drugs is in young patients at the early stages of the disease for the relief of mild movement symptoms particularly tremors and muscle stiffness. Anticholinergic drugs play more of a role in tremor-predominant PD, where they may be used as monotherapy in the early stages. However, when anticholinergics are used, they are usually done so in combination with levodopa and the other aforementioned medications. They are generally avoided in elderly patients or those with cognitive problems, risk of confusion with this class of drugs. Tablet and oral suspension preparations exist. Examples of anticholinergics include benztrapine, orphenadrine, procyclidine, and trihexyphenidyl (Benzhexol).

Amantadine

Doctors may prescribe amantadine alone to provide short-term relief of symptoms of mild, early-stage Parkinson's disease. It may also be given with carbidopa-levodopa therapy during the later stages of Parkinson's disease to control involuntary movements (dyskinesia) induced by carbidopa-levodopa. Side effects may include a purple mottling of the skin, ankle swelling, or hallucinations.

Other medicines used to treat Parkinson's^[10] include Dopamine agonists to mimic the role of dopamine in the brain, MAO-B inhibitors to slow down an enzyme that breaks down dopamine in the brain, COMT inhibitors to help break down dopamine, amantadine an old antiviral drug, to reduce involuntary movements, and anticholinergic to reduce tremors and muscle rigidity.

The current medication treatments for Parkinson's disease have a variety of negative effects. As a result, herbal remedies should be considered as an alternate treatment option. Plants have been used as a source of medicine since the dawn of humanity. Plants have served humans as useful components of medications, seasonings, beverages, cosmetics, and dyes for thousands of years, and have played an important part in sustaining human health and improving the quality of life. Because *Malaxis acuminata* has traditionally been used to treat neurodegenerative illnesses (including Parkinson's disease), this plant could be useful in the treatment of PD. Using the SH-SY5Y cell line, the current work attempts to examine *in-vitro* screening of anti-activity Parkinson's of the entire *Malaxis acuminata* plant.^[10-12]

Plant profile of *Malaxis acuminata*^[13,14]

Distribution

The *Malaxis* genus is found all over the world. It can be found in India, Bangladesh, the Eastern Himalayas, Lower India, Nepal, the Western Himalayas, Bhutan,

the Andaman Islands, Myanmar, Thailand, Malaysia, Laos, Cambodia, China, Vietnam, Java, Sumatra, and the Philippines. It can also be found in upper mountain grasslands in Ecuador and Peru, at elevations of 1500-3850 meters. *M. acuminata* is a Himalayan medicinal orchid that is rare, perennial, and endangered. It can be found in the Himalayan regions temperate to subalpine zones. *M. acuminata* is found in abundance in Uttarakhand. The most significant pseudobulb of this plant is well known in the Indian System of Medicine for its therapeutic qualities and has been commercialized under the name Jeevak since time immemorial.

Description

In April, the *M. acuminata* plant begins to grow from seed germination or a latent apical bud from the subsurface section. The plant is perennial and spends the first two or more years of its life in the vegetative phase. The plant has two sessile, unequally sized orbicular-ovate to ovate-lanceolate leaves. The plant forms its reproductive flower-bearing axis^{12,13} during its third or later year of growth. Inflorescences can grow up to 10-15 cm in length. Flowers have a diameter of 3 mm and are yellowish-green in colour. The bracts are lanceolate and shorter than the ovary, with sub-equal sepals and oblong-lanceolate, sub-acute sepals. Petals are shorter and more linear than sepals. Lips are oval and sharply pointed, with thickened edges. July-August is when the flowers bloom. Tuber-round, gleaming, with a stem that resembles a bovine horn in shape and curve. The flavor is slightly bitter and has a fat-like consistency. Ruthisha P. K. *et al.* explored the qualitative and quantitative evaluation of the principal bioactive elements of *Seidenfia rheedii* (Sw.) Szlach, a medicinally important plant. Whole plant extract in water, ethanol, chloroform, and petroleum ether. The sample contained carbohydrates, proteins, amino acids, saponins, flavonoids, terpenoids, glycosides, tannins, alkaloids, and phenols.^[15,16] Renjini Haridas *et al.* used the DPPH, ABTS•+, and ferrous ion chelating assays to investigate the antioxidant activity of the whole plant extract. The crude methanol and ethyl acetate extracts of *M. rheedii* showed higher amounts of ferrous ion chelating activity. In DPPH activity and ABTS•+ activity, petroleum ether extract had the highest antioxidant activity. The findings indicate that *M. rheedii* has promising antioxidant activity and could be used as a natural antioxidant source.^[17] The antioxidant activity of butanol extract of *Malaxis acuminata* was investigated using various methods, including 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, reduction capability by Fe³⁺-Fe²⁺ transformation method, and hydrogen

peroxide scavenging method, according to Pushpa Sharma *et al.* The extract was discovered to have high antioxidant activity.^[18] Ashish Gupta *et al.* have reported that the *Malaxis acuminata* is used for the preparation of Chyavanprash and tonics. The tonic is energizing, cures tuberculosis, and enhances sperm formation.^[19]

Screening Method

In vivo METHOD^[10,20]

- MPTP model of Parkinson's disease
- Reserpine antagonism
- Elevated body swing test
- Skilled test in rats
- Gait analysis
- Stepping test in rats
- Transfer of glial cell line-derived neurotrophic factor (GDNF) *in vitro* Method^[12]
- Experiment using rat strial slices
- Dopamine stimulated adenyl cyclase activity
- Cultures of *Substantia nigra*
- Inhibition of Apoptosis in neuroblastoma SH-SY5Y cells

MATERIALS AND METHOD

The whole plant of *Malaxis acuminata* was collected from Yercurd in December-2020. The plant has been taxonomically identified and authenticated by the botanist Dr. S. Radha. MSc. Ph.D. Central Siddha Medicinal plant garden Mettur dam. Tamilnadu. The authenticated plants were used for the preparation of the extract. The authenticated plant was used for the extracts.

Preparation of the extracts

The collected, cleaned and powdered whole plant of *Malaxis acuminata* was used for extraction purposes. In the soxhlet apparatus, the material was equally packed. It was then extracted with various solvents from non-polar to polar such as Petroleum ether aqueous and ethanol. Continuous hot percolation process was used as extraction method using petroleum ether (60-80°C), alcohol 90% v/v. (75-78°C), and distilled water as solvents.

In vitro neuroprotective effect determination by MTT assay

Instruments and reagents used

DMEM media (Sigma Aldrich, USA D5648), Fetal bovine serum (Gibco, US origin), 0.25% Trypsin (Invitrogen, USA 25200-056), Micropipettes (F1 Thermo scientific USA), CO₂ incubator (Eppendorf,

GERMANY), Phase Contrast Microscope (Olympus, JAPAN with Optika Pro 5 Camera), MTT (Sigma Aldrich M5655), ELISA Reader (ERBA, GERMANY), Culture Plates and Flasks (NUNC, Thermo scientific USA)

PROCEDURE

NCCS provided the SHSY-5Y (Neuroblastoma cells) cell line, was kept in Dulbecco's Modified Eagles medium (DMEM) from the National Centre for Cell Sciences (NCCS), Pune, India, (Sigma Aldrich, USA). The cell line was grown in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution comprising Penicillin (100U/ml), Streptomycin (100g/ml), and Amphotericin B (2.5g/ml) in a 25 cm² tissue culture flask. In a humidified 5 percent CO₂ incubator, cultured cell lines were incubated at 37°C (NBS Eppendorf, Germany). The vitality of the cells was determined by using an inverted phase-contrast microscope to observe the cells directly, followed by the MTT assay method.

Cells seeding in 96 well plates

A two-day-old confluent monolayer of cells was trypsinized and suspended in 10% growth media. A 100l cell suspension (5x10⁴ cells/well) was seeded in a 96-well tissue culture plate and cultured at 37°C in a humidified 5% CO₂ incubator.

Preparation of compound stock: Using a cyclomixer, 1mg of the sample was weighed and thoroughly dissolved in 1mL DMEM. To assure sterility, the extract solution was filtered using a 0.22 m Millipore syringe filter. To induce toxicity, rotenone (10M) was applied.

Cytotoxicity Evaluation

After achieving sufficient growth, Rotenone (10M) was added to induce toxicity and incubated for one hour. Prepared extracts in 5% DMEM were serially diluted five times by two-fold dilution (100g, 50g, 25g, 12.5g, and 6.25g in 500l of 5% DMEM) and each concentration of 100l was added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO₂ incubator. The formula was used to calculate the percentage of growth inhibition:

$$\% \text{ of Viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Cytotoxicity Assay by Direct Microscopic observation

The entire plate was seen in an inverted phase-contrast tissue culture microscope (Olympus CKX41 with

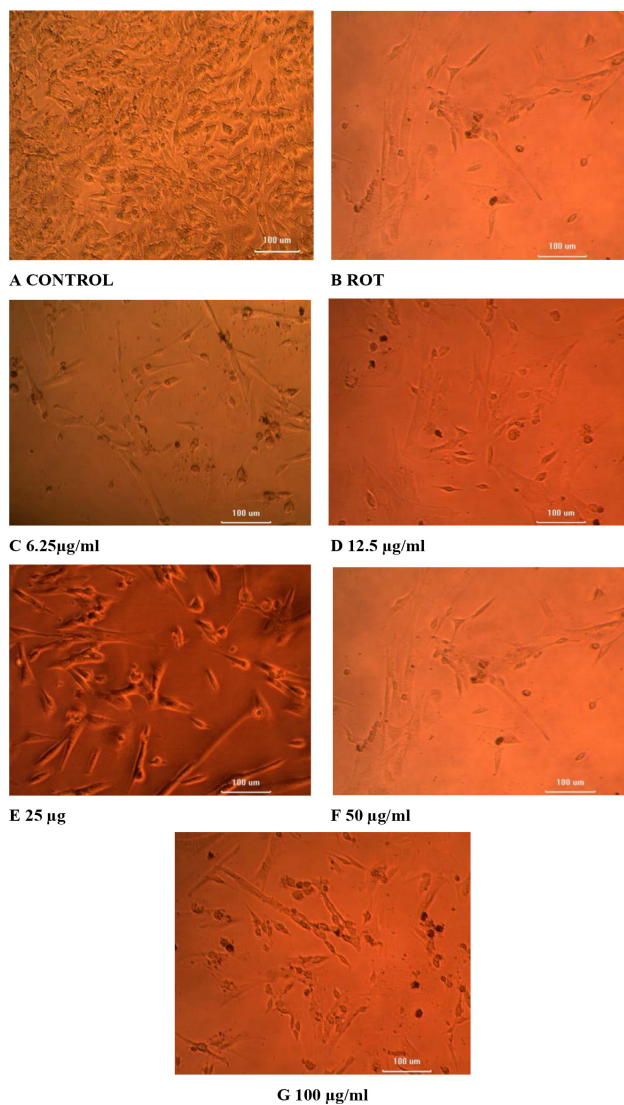


Figure 1: *In vitro* anti-Parkinson's activity of ethanolic extract of *Malaxis acuminata* against SH-SY5Y cell line.

- A- Normal SH-SY5Y
- B- Rotenone induced toxicity
- C- SH-SY5Y cell line treated with 6.25 µg/ml of ethanolic extract of *Malaxis acuminata*
- D- SH-SY5Y cell line of treated with 12.5 µg/ml of ethanolic extract of *Malaxis acuminata*
- E SH-SY5Y cell line of treated with 25 µg/ml of ethanolic extract of *Malaxis acuminata*
- F- SH-SY5Y cell line of treated with 50 µg/ml of ethanolic extract of *Malaxis acuminata*
- G- SH-SY5Y cell line of treated with 100 µg/ml of ethanolic extract of *Malaxis acuminata*

Optika Pro5 CCD camera) every 24 hr for up to 72 hr, and microscopic observations were recorded as pictures, as shown in Figure 1 Any visible changes in cell shape, such as rounding or shrinkage of cells, granulation, and vacuolization in the cytoplasm, were deemed cytotoxicity indications.

Cytotoxicity Assay by MTT Method

15 mg MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved, and sterilized by filter sterilization. After a 24 hr incubation period, the sample content in the wells was removed, and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken, and the plate was incubated for 4 hr at 37°C in a humidified 5 percent CO₂ incubator. The supernatant was removed after the incubation period, and 100µl of MTT Solubilization Solution (DMSO) was added, and the wells were gently agitated by pipetting up and down to solubilize the formazan crystals.^[21]

RESULTS

Because rotenone did not produce any reactive oxygen species, it is plausible that it inhibited enzymes involved in glutathione synthesis or formed complexes with glutathione, lowering intracellular free reduced glutathione levels. It's also possible that rotenone exposure resulted in intracellular glutathione depletion through membrane leakage. By using different concentrations in the MTT assay the ethanolic extract of *Malaxis acuminata* was used to treat the SH-SY5Y cell line. The cell percentage viability decreased gradually when the doses have been increasing in 24Hr. The most effective concentration to inhibit cell growth was found to be 6.25µg/ml. As the concentration of the extract increases, no of viable cells decreased. Using varied concentrations of 6.25, 12.5, 25, 50, and 100g/ml, the percentage of various cell viability was determined to be 51.9%, 61.09 percent, 70.85 percent, 63.66 percent, and 59.92 percent. The vitality of rotenone cells was shown to be 47.65 percent. The ethanolic extracts reduced rotenone-induced Parkinson's disease activity. The results are graphically presented in Table 1 and Figure 2, respectively.

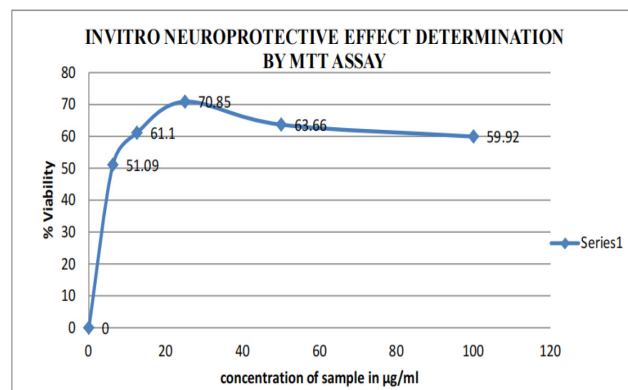


Figure 2: *In-vitro* neuroprotective effect determination by MTT Assay.

Table 1: *In vitro* neuroprotective effect determination by MTT Assay

Sample Concentration($\mu\text{g}/\text{ml}$)	OD I	OD II	OD II1	Average Absorbance @ 540nm	Percentage Viability
CONTROL	0.3275	0.3186	0.3237	0.3233	100
Rotenone	0.1492	0.1549	0.1581	0.1541	47.65
6.25	0.1644	0.1686	0.1625	0.1652	51.09
12.5	0.1988	0.1942	0.1996	0.1975	61.10
25	0.2297	0.2307	0.2268	0.2291	70.85
50	0.2016	0.2133	0.2025	0.2058	63.66
100	0.1892	0.1954	0.1966	0.1937	59.92

DISCUSSION

Using the SH-SY5Y Neuroblastoma cell line, the study wanted to see how ethanolic extracts of *Malaxis acuminata* plants affected rotenone-induced neuronal damage. The plant extracts were tested *in vitro* for cytotoxicity, intracellular redox state (ROS and intracellular glutathione concentration), MMP, and caspase-3 activity. Because rotenone did not produce any reactive oxygen species, it is plausible that it inhibited enzymes involved in glutathione synthesis or formed complexes with glutathione, lowering intracellular free reduced glutathione levels. It's also possible that rotenone exposure resulted in intracellular glutathione depletion through membrane leakage.

The ethanolic extract of *Malaxis acuminata* was utilised to treat the SH-SY5Y cell line using varied concentrations in the MTT experiment. When the doses were steadily increased over 24 hr, the percentage viability of the cells fell. 6.25g/ml was shown to be the most effective concentration for inhibiting cell proliferation. The number of viable cells dropped as the extract concentration increased. Using varied concentrations of 6.25, 12.5, 25, 50, and 100g/ml, the percentage of various cell viability was determined to be 51.9%, 61.09 percent, 70.85 percent, 63.66 percent, and 59.92 percent. Rotenone was discovered to have a cell viability of 47.65 percent. Rotenone-induced activity of Anti-was Parkinson's reduced by ethanolic extracts. The results are represented graphically in Table 1 and Figure 2 correspondingly.

CONCLUSION

We inferred from the findings that the ethanolic extract had anti-Parkinson's action and restored intracellular

glutathione levels after rotenone treatment, implying that they may have neuroprotective qualities. The plant has good anti-Parkinson's activity by using the SH-SY5Y cell line *in vitro*. In future studies *in vivo method* can be done to prove the anti-Parkinson's effect of *Malaxis acuminata* on animal models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

PD: Parkinson's disease; **MAO-B:** Monoamine oxidase type B; **DMEM:** Dulbecco's Modified Eagles medium; **SNpc:** Substantia Nigra Pars Compacta; **aSyn:** Alpha Synuclein; **MMP:** Mitochondrial membrane potential; **PBS:** Phosphate buffered saline.

SUMMARY

The aim of the study was to determine the effects of ethanolic extracts of *Malaxis acuminata* plants on rotenone-induced neuronal toxicity using the SH-SY5Y Neuroblastoma cell line. Most effective concentration to inhibit the cell growth was found to be 6.25 $\mu\text{g}/\text{ml}$. Following rotenone treatment, ethanolic extract showed anti-action Parkinson's and restored intracellular

glutathione concentration, implying that they may have neuroprotective characteristics. In the future, *in vivo* investigations on animal models could be used to show the Antri-impact Parkinson's of *Malaxis acuminata*.

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