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Neuroprotective effects of the standardized extract of Centella asiatica ECa233 in rotenone-induced parkinsonﬀs disease model

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NEUROPROTECTIVE EFFECTS OF THE STANDARDIZED EXTRACT OF CENTELLA ASIATICA ECA233 IN ROTENONE-INDUCED PARKINSON'S DISEASE MODEL

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ฤทธิ์ปกป้องประสาทของสารสกัดมาตรฐานบัวบก อีซีเอ233 ในแบบจำลองโรคพาร์กินสันที่เหนี่ยวนำโดยโรตีโนน

นายนฤดล ธีรภัทรกานต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	NEUROPROTECTIVE EFFECTS OF THE STANDARDIZED EXTRACT OF CENTELLA ASIATICA ECA233 IN ROTENONE-INDUCED PARKINSON'S DISEASE MODEL
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นฤดล ธีรภัทรกานต์ : ฤทธิ์ปกป้องประสาทของสารสกัดมาตรฐานบัวบก อีซีเอ233 ในแบบจำลองโรคพาร์กินสันที่เหนี่ยวนำโดยโรติโนน . (
NEUROPROTECTIVE EFFECTS OF THE STANDARDIZED EXTRACT OF CENTELLA ASIATICA ECA233 IN ROTENONE-INDUCED PARKINSON'S DISEASE MODEL) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร.รัชนี รอดศิริ, อ.ที่ปรึกษาร่วม : ผศ. ภญ. ดร.วริษา พงศ์เรขนานนท์

สารสกัดมาตรฐานบัวบก อีซีเอ233 ประกอบด้วยสารสำคัญ asiaticoside และ madecassoside การศึกษาก่อนหน้ารายงานฤทธิ์ทางเภสัชวิทยาของอีซีเอ233 โดยเฉพาะอย่างยิ่งการรักษาความผิดปกติของระบบประสาทส่วนกลาง วัตถุประสงค์ของการศึกษานี้คือเพื่อทดสอบฤทธิ์ปกป้องประสาทและกลไกการปกป้องประสาทของอีซีเอ233 ในหนูแรทและในเซลล์ประสาท SH-SY5Y ที่เหนี่ยวนำให้เกิดความเป็นพิษด้วยโรติโนนที่เป็นแบบจำลองโรคพาร์กินสัน การศึกษาในสัตว์ทดลอง ใช้หนูแรทเพศผู้สายพันธุ์ Wistar โดยแบ่งหนูแรทออกเป็น 5 กลุ่ม ได้แก่ กลุ่มควบคุม, PD, PD-ECa10, PD-ECa30 และ PD-ECa100 วันที่ 1-20 หนูกลุ่มควบคุมและกลุ่ม PD จะป้อน 0.5% carboxymethylcellulose (CMC) ปริมาตร 1 มล./กก. ส่วนหนูกลุ่ม PD-ECa10, PD-ECa30 และ PD-ECa100 จะได้รับอีซีเอ233 ขนาด 10, 30 และ 100 มก./กก. ตามลำดับ โดยการป้อนในวันที่ 15-20 หนูกลุ่ม PD และ PD-ECa จะได้รับโรติโนนขนาด 2.5 มก./กก. ฉีดทางช่องท้อง ส่วนหนูกลุ่มควบคุมจะได้รับ 2% dimethlysulfoxide (DMSO) ฉีดทางช่องท้อง ทำการทดสอบพฤติกรรมการเคลื่อนที่ ในวันที่ 1, 14, 17 และ 20 ในวันที่ 21 หนูทุกตัวทำการทดสอบ apomorphine challenge เมื่อสิ้นสุดการทดลองจะเก็บสมองของหนูเพื่อวิเคราะห์เซลล์ประสาทโดปามีนในวิถีประสาท nigrostriatal การทำงานของไมโตคอนเดรีย complex I ระดับ malondialdehyde (MDA) การแสดงออกของเอนไซม์ superoxide dismutase (SOD) และ catalase ผลการทดลองแสดงให้เห็นว่าหนูกลุ่ม PD มีพฤติกรรมในการเคลื่อนที่ลดลง แต่การให้อีซีเอ233 (30 มก./กก.) สามารถปกป้องความผิดปกติของการเคลื่อนที่ในหนูกลุ่ม PD-ECa30 ได้อย่างมีนัยสำคัญทางสถิติ ($p<0.01$) ในวันที่ 21 apomorphine ทำให้พฤติกรรมการเคลื่อนที่ดีขึ้น แสดงให้เห็นว่าโรติโนนมีผลลดระดับโดปามีนในวิถีประสาท nigrostriatal อีซีเอ233 (30 มก./กก.) ปกป้องการตายของเซลล์ประสาทโดปามีนในสมองส่วน substantia nigra ($p<0.001$) และเพิ่มเส้นใยประสาทโดปามีนที่สมองส่วน striatum ($p<0.001$) นอกจากนี้อีซีเอ233 (30 มก./กก.) ลดระดับ MDA ในสมอง ($p<0.05$) และเพิ่มการทำงานของไมโตคอนเดรีย complex I ($p<0.05$) การแสดงออกของเอนไซม์ SOD และ catalase ($p<0.05$) การทดลองในเซลล์ SH-SY5Y เพื่อศึกษากลไกการออกฤทธิ์ในระดับโมเลกุล โดยให้อีซีเอ233 (5, 10, 25, 50 และ 100 มคก./มล.) แก่เซลล์ SH-SY5Y เป็นเวลา 12 ชม. ตามด้วยโรติโนน 48 ชม. จากนั้นวัดการรอดชีวิตของเซลล์ ความต่างศักย์ของเยื่อหุ้มไมโตคอนเดรีย และการเกิด mitophagy ด้วยเทคนิค resazurin, tetramethylrhodamine, ethyl ester (TMRE) และ mitophagy dye ตามลำดับ การวัดระดับอนุมูลอิสระภายในเซลล์ด้วยเทคนิค dichloro-dihydro-fluorescein diacetate (DCFH-DA) จะให้อีซีเอ233 แก่เซลล์ เป็นระยะเวลา 12 ชม. แล้วให้โรติโนน 6 หรือ 24 หรือ 48 ชม. การวัดการแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการ autophagy และการตายของเซลล์แบบ apoptosis จะให้อีซีเอ233 แก่เซลล์เป็นเวลา 12 ชม. ตามด้วยโรติโนนเป็นเวลา 24 ชม. ผลการทดลองพบว่า อีซีเอ233 (10-100 มคก./มล.) เพิ่มการรอดชีวิตของเซลล์ เมื่อเปรียบเทียบกับกลุ่มที่ได้รับโรติโนนเพียงอย่างเดียว ($p<0.01$) อีซีเอ233 (50-100 มคก./มล.) ลดระดับอนุมูลอิสระภายในเซลล์ที่ 24 ชม. หลังได้รับ rotenone ($p<0.05$) แต่ไม่พบผลดังกล่าวที่เวลา 6 และ 48 ชม. นอกจากนี้อีซีเอ233 (25-100 มคก./มล.) ปกป้องการลดลงของความต่างศักย์ของเยื่อหุ้มไมโตคอนเดรีย ($p<0.01$) และ mitophagy ($p<0.05$) ที่เหนี่ยวนำโดยโรติโนน อีซีเอ233 (50-100 มคก./มล.) เพิ่มการแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการ autophagy ได้แก่ Beclin-1 และ LC3II/I ($p<0.05$) นอกจากนี้ซีเอ233 (25-100 มคก./มล.) เพิ่มการแสดงออกของ Bcl-2 ($p<0.05$) และลดสัดส่วนของ cleaved-caspase3/caspase3 ($p<0.05$) โดยสรุปอีซีเอ233 ปกป้องความเป็นพิษต่อเซลล์ประสาทที่ถูกเหนี่ยวนำโดยโรติโนนทั้งในสัตว์ทดลองและในหลอดทดลอง กลไกการปกป้องประสาทของอีซีเอ233 ได้แก่ การปกป้องการทำงานของไมโตคอนเดรีย complex I ลดการเกิด lipid peroxidation เหนี่ยวนำเอนไซม์ต้านอนุมูลอิสระ ปกป้องเยื่อหุ้มไมโตคอนเดรีย เพิ่มกระบวนการ mitophagy และปกป้องการตายของเซลล์แบบ apoptosis จากผลการศึกษาแสดงให้เห็นว่าอีซีเอ233 มีศักยภาพที่จะพัฒนาเป็นยารักษาโรคพาร์กินสันในอนาคต

สาขาวิชา	เภสัชวิทยา	ลายมือชื่อนิสิต .....
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Narudol Teerapattarakan :
NEUROPROTECTIVE EFFECTS OF THE STANDARDIZED EXTRACT OF CENTELLA ASIATICA ECA233 IN ROTENONE-INDUCED PARKINSON'S DISEASE MODEL . Advisor: Asst. Prof. RATCHANEE RODSIRI, Ph.D. Co-advisor: Asst. Prof. VARISA PONGRAKHANANON, Ph.D.

The standardized extract of *Centella asiatica* ECa233 consists of asiaticoside and madecassoside. Previous studies reported various pharmacological effects of, ECa233, especially in the central nervous system disorders. The present study aimed to investigate the neuroprotective effects and protective mechanisms of ECa233 against rotenone-induced neurotoxicity in rats and in neuroblastoma SH-SY5Y cells. In animal study, male Wistar rats were divided into five experimental groups (n=10); control, PD, PD-ECa10, PD-ECa30, and PD-EC100. On days 1-20, control and PD rats received 0.5% carboxymethylcellulose (CMC) (1 mL/kg, p.o.) while ECa233 10, 30 and 100 mg/kg (p.o.) were given to PD-ECa10, PD-ECa30, and PD-EC100, respectively. Rotenone (2.5 mg/kg, i.p.) was given to PD and PD-ECa groups on days 15-20 while controls received 2% Dimethylsulfoxide (DMSO) (i.p.) Locomotor activity was performed on days 1, 14, 17, and 20, and apomorphine challenge was tested on day 21. Brains were collected for further analysis of tyrosine hydroxylase (TH)-positive neurons in the nigrostriatal pathway, mitochondrial complex I activity, malondialdehyde (MDA) levels and superoxide dismutase (SOD) and catalase protein expression. The results showed that locomotor activity of rats was significantly reduced, while ECa233 (30 mg/kg) pretreatment significantly alleviated motor deficits in PD-ECa233 compared to PD rats on day 20 ($p<0.01$). On day 21, the locomotor deficit was responded to apomorphine challenge, suggesting dopamine depletion in the nigrostriatal pathway. ECa233 (30 mg/kg) protected against rotenone-induced dopaminergic neuronal loss in the substantia nigra ($p<0.001$) and preserved dopaminergic axonal fibers in the striatum ($p<0.0001$). ECa233 (30 mg/kg) also decreased brain MDA levels ($p<0.05$) while increased mitochondrial complex I activity ($p<0.05$) and SOD and catalase protein expression ($p<0.05$). To investigate the molecular mechanisms of ECa233, SH-SY5Y cells were treated with ECa233 (10, 25, 50, and 100 µg/mL) for 12 h, followed by rotenone for 48 h. Cell viability, mitochondrial membrane potential, and mitophagy were measured using resazurin assay, tetramethylrhodamine, ethyl ester (TMRE) assay, and mitophagy detection, respectively. To determine intracellular reactive oxygen species (ROS) levels by dichloro-dihydro-fluorescein diacetate (DCFH-DA) technique, cells were treated with ECa233 for 12 h followed by rotenone for either 6, 24 or 48 h. Autophagy and apoptosis-associated proteins were measured after 12 h ECa233 pretreatment followed by 24 h rotenone incubation. The results showed that ECa233 (10-100 µg/mL) significantly increased cell viability compared to rotenone-treated alone ($p<0.01$). ECa233 (50-100 µg/mL) decreased intracellular ROS levels in rotenone-treated cells at 24 ($p<0.05$), but not 6 and 48 h. In addition, ECa233 (25-100 µg/mL) prevented against rotenone-induced the reduction of mitochondrial membrane potential ($p<0.01$) and mitophagy ($p<0.05$). The expressions of autophagic-associated proteins, Beclin-1 and LC3II/I, were significantly increased in ECa233 (50-100 µg/mL) pretreatment groups ($p<0.05$). In addition, ECa233 (25-100 µg/mL) significantly increased Bcl-2 expression ($p<0.05$) while decreased cleaved-caspase-3/caspase-3 ratio ($p<0.05$). To summarize, ECa233 protects against rotenone-induced neurotoxicity *in vivo* and *in vitro*. The underlying neuroprotective mechanisms are mitochondrial complex I protection, lipid peroxidation reduction, induction of antioxidant enzymes, prevention of mitochondria membrane potential reduction, enhancing mitophagy, and prevention of neuronal apoptosis. Taken together, the current study suggests that ECa233 is a potential compound in drug development for the treatment of Parkinson's disease.

Field of Study: Pharmacology Student's Signature .....
Academic Year: 2018 Advisor's Signature .....
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Narudol Teerapattarakan

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ABBREVIATIONS

α = alpha

β = beta

°C = degree Celsius

< = less than

μL = microliter(s)

μM = micromolar(s)

/ = per

% = percentage

± = plus-minus sign

x g = relative centrifugal force

6-OHDA = 6-hydroxydopamine

AADC = aromatic amino acid decarboxylase

Abs = absorbance

ANOVA = analysis of variance

ATP = adenosine triphosphate

Bax = Bcl-2 associated X protein

Bcl-2 = B-cell lymphoma-2

BBB = blood brain barrier

BSA = bovine serum albumin

Ca^{2+} = calcium ion

CMC = carboxy methyl cellulose

CNS = central nervous system

CO_2 = carbon dioxide

Complex I = complex one of mitochondrial respiratory chain

COMT = catechol-O-methyltransferase

CYP450 = cytochrome P450

DAT = dopamine transporter

DJ-1 = nucleic acid deglycase DJ-1

DMSO = dimethyl sulfoxide

g = gram(s)

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

h = hour(s)

H_2O_2 = hydrogen peroxide

IC_{50} = half maximal inhibitory concentration

iNOS = inducible nitric oxide synthase

i.p. = intraperitoneal injection

kg = kilogram(s)

LBs = Lewy bodies

LC3 = microtubule-associated protein light chain 3

L-dopa = levodopa

LRRK2 = Leucine-rich repeat kinase 2

M = Molarity

MAO_B = monoamine oxidase subtype B

MDA = malondialdehyde

mg = milligram(s)

mL = milliliter(s)

MPP+ = 1-methyl-4-phenylpyridinium

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

n = number

Na_2CO_3 = sodium carbonate

NADH = nicotinamide adenine dinucleotide

nM = nanoMolar

NMDA = N-methyl-D-aspartate

NO = nitric oxide

PARK2 = Parkin RBR E3 ubiquitin protein ligase

PBS = phosphate-buffered saline

P-gp = P-glycoprotein

pH = potential of hydrogen ion

PINK1 = PTEN-induced putative kinase 1

PTP = permeability transition pores

p.o. = per os, oral administration

ROS = reactive oxygen species

s.c. = subcutaneous injection

SDS = sodium dodecyl sulfate

S.E.M. = standard error of mean

SNCA = Synuclein alfa

SNpc = substantia nigra pars compacta

SOD = superoxide dismutase

TBA = thiobarbituric acid

TBARS = thiobarbituric acid reacting substances

TH = tyrosine hydroxylase

TNF-α = tumor necrosis factor alpha

TMRE = tetramethylrhodamine ethyl ester

VDAC = voltage-dependent anion channel

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Parkinson's disease is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the nigrostriatal pathway Johnson and Bobrovskaya (2015). The loss of dopaminergic cells subsequently causes motor symptoms, including resting tremor, bradykinesia, rigidity, and postural imbalance. The current managements of Parkinson's disease aim to increase brain dopamine levels and to alleviate motor symptoms in Parkinson's disease patients. However, none of these medicines can slow or stop disease progression (Sharma et al., 2015). Therefore, finding the novel therapeutic agents for Parkinson's disease is still required.

Many studies revealed causes of dopaminergic neuronal death including oxidative stress, neuroinflammation, and mitochondria dysfunction (Gaki & Papavassiliou, 2014; Johnson & Bobrovskaya, 2015; Wang et al., 2015). Mitochondrial dysfunction has gained more interest as it is presented in the pathogenesis of sporadic and familial types of Parkinson's disease (Winklhofer & Haass, 2010). The inhibition of mitochondrial complex I results in the disruption of electron transport chain causing the reduction of ATP levels, reactive oxygen species production, oxidative stress and neuronal cell death (Winklhofer & Haass, 2010). Since mitochondria dysfunction leads to molecular events of cell death, cells undergo mitophagy process to protect themselves. Mitophagy is a subset of macro-autophagy aiming to maintain cellular homeostasis by increasing the clearance of the damaged mitochondria (Anding & Baehrecke, 2017; Rodriguez-Enriquez et al., 2006). Therefore,

the therapeutic strategy targeting mitochondria function is one of the promising therapeutic approach in Parkinson's disease.

Rotenone, a mitochondrial complex I inhibitor, was used to generate Parkinson's disease model in this study. Rotenone disrupts mitochondrial electron transport chain causing loss of ATP, reactive oxygen species generation and neuronal cell death (Johnson & Bobrovskaya, 2015; Wrangel et al., 2015). Rotenone causes selective nigrostriatal degeneration and produce parkinsonism-liked behaviors in animals (Sherer et al., 2003a; Testa et al., 2005). Rotenone also inhibits mitophagy and promotes **α**-synuclein aggregation *in vitro* (Wu et al., 2015). By using rotenone-induced neurotoxicity, this study aimed to elucidate the neuroprotective mechanisms involving mitochondria protection, mitophagy enhancement, and antioxidant.

Centella asiatica is a tropical medicinal plant wildly used in the South East Asia. In Thailand, *Centella asiatica* has been approved to use externally as a wound healing agent according to the National list of essential medicines of Thailand (Thai-FDA, 2012). The major active ingredients of *Centella asiatica* are asiaticoside and madecassoside (Günther & Wagner, 1996). Previous scientific evidences have reported various pharmacological effects of *Centella asiatica* such as gastroprotection (Cheng & Koo, 2000), wound healing effect (Somboonwong et al., 2012), anti-inflammatory effect (George et al., 2009), cognitive improvement (Soumyanath et al., 2012) and neuroprotective effect (Haleagrahara & Ponnusamy, 2010). However, the crude extracts of *Centella asiatica* generally vary in their compositions contributing to the uncertain biological responses. In order to minimize those unpredictably pharmacological effects of crude extracts, the standardized extract of *Centella asiatica* ECa233 has been established by Faculty of Pharmaceutical Sciences, Chulalongkorn University. ECa233

consists of madecassoside and asiaticoside not less than 80%. The ratio between madecassoside and asiaticoside are 1.5:1, respectively (Saifah E, 2009). Previous studies have revealed the anxiolytic effect (Wanasuntronwong et al., 2012), the neuritogenic effect (Wanakhachornkrai et al., 2013)and learning and memory improvement of ECa233 (Tantisira et al., 2010). Although ECa233 exerts various pharmacological effects in the central nervous system (CNS), the neuroprotective effect of ECa233 in Parkinson's disease model has not been clarified. Therefore, this study aimed to demonstrate the neuroprotective effects of Eca233 against rotenone-induced Parkinson's disease model and to investigate the mechanisms underlying the neuroprotective effects of ECa233.

1.2 Objectives

1. To investigate the neuroprotective effects of the standardized extract of *Centella aisiatica* ECa233

2. To elucidate the mechanisms underlying the neuroprotective effects of the standardized extract of *Centella aisiatica* ECa233

1.3 Research questions

1. Does ECa233 produce the neuroprotective effects in the rotenone model of Parkinson's disease?

2. What are the mechanisms underlying the neuroprotective effects of ECa233?

1.4 Hypothesis

ECa233 shows the neuroprotective effects in the *in vivo* and *in vitro* models of Parkinson's disease induced by rotenone. The mechanisms underlying the neuroprotective effects of ECa233 are mediated through antioxidant activity, mitochondrial protection, and mitophagy enhancement.

1.5 Research design

Experimental Research

1.6 Benefits of the study

This study provides the scientific information of ECa233, which supports further development of ECa233 as a novel therapeutic agent in Parkinson's disease.

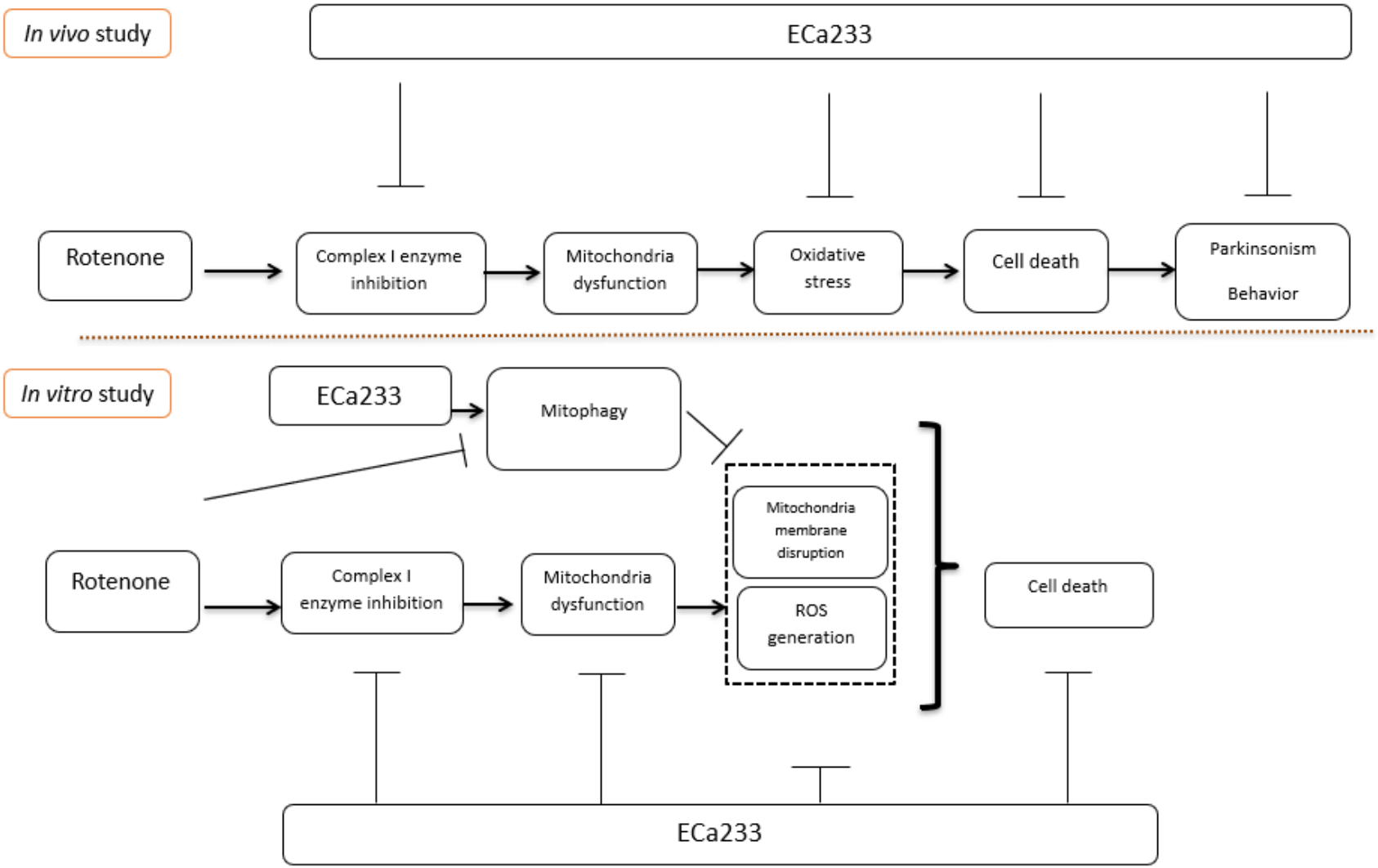


Figure 1 Conceptual framework

CHAPTER 2
LITERATURE REVIEW

2.1 Parkinson's disease

Parkinson's disease is the second most prevalent neurodegenerative disorder worldwide with an estimated prevalence of 315/100,000 among the population aged over 40 (Pringsheim et al., 2014). In Thailand, the prevalence of Parkinson's disease was 425/100,000 among the population (Bhidayasiri et al., 2011). Parkinson's disease can be classified into two types, which are familial and sporadic types. Familial Parkinson's disease is usually early onset and caused by mutations of many genes such as *SNCA*, *PARK2, PINK1, DJ-1*, and *LRRK2* (Ferreira & Massano, 2017). These genes regulate protein-homeostasis, mitochondria function, and antioxidant mechanisms (Ryan et al., 2015; Simon-Sanchez et al., 2009). Sporadic Parkinson's disease is normally late onset. Although the causes of sporadic Parkinson's disease remain unclear, many evidences have shown that aging, environmental toxin, and genetic mutation are associated with sporadic Parkinson' s disease (Billingsley et al., 2018; McCormack et al., 2002) **(Figure 2)**.

The cardinal motor symptoms of Parkinson's disease include resting tremor, bradykinesia, rigidity, and postural instability. Although Parkinson's disease has been recognized as a motor-related disorder, neuropsychiatric and non-motor manifestations are also presented, especially in advanced stage of disease (Barone et al., 2009). Non-motor symptoms are caused by dysfunctional adrenergic, cholinergic, and serotonergic systems (Chaudhuri & Schapira, 2009). These non-motor symptoms include cognitive dysfunctions, hallucinations, mood disorders, sleep disorders, loss of smell sensation, and gastrointestinal disturbances (Chaudhuri & Schapira, 2009).

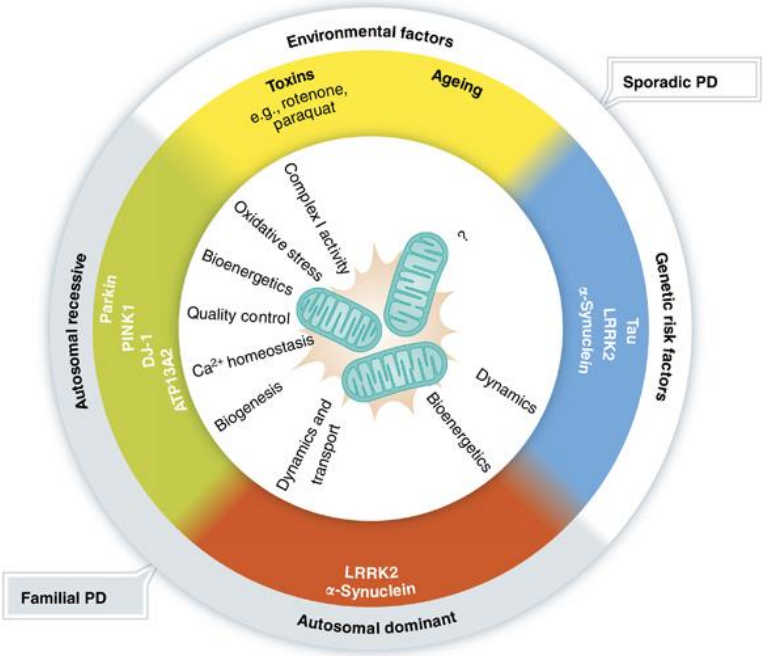


Figure 2 Classification of Parkinson's disease (Fu et al., 2015).

The current pharmacological management of Parkinson's disease aims to increase dopamine levels in the brain and subsequently relieve motor symptoms. Drugs used in Parkinson's disease can be classified into six classes based on their mechanisms of action which are levodopa, dopamine receptor agonist, monoamine oxidase-B (MAO-B) inhibitor, catechol-*O*-methyltransferase (COMT) inhibitor, dopamine releaser and anticholinergic drug **(Figure 3)**. Levodopa is an intermediate precursor of dopamine which is converted to dopamine by aromatic amino acid decarboxylase (AADC). Levodopa is usually administered in combination with AADC inhibitors, such as benserazide and carbidopa, to prevent peripheral metabolism of levodopa. Dopamine receptor agonist directly stimulates dopamine receptor in the striatum and therefore mimic the endogenous effect of dopamine. They can be used as monotherapy in early Parkinson's disease or in combination with levodopa to manage

motor fluctuation. COMT inhibitor, entacapone, inhibits the peripheral degradation of levodopa; thus, increases levodopa level in the brain. MAO-B-inhibitors can be used as monotherapy and in combination with levodopa. MAO-B-inhibitors inhibit dopamine degradation; therefore, they can extend the effects of dopamine in the synapse. Anticholinergic drugs are used to keep the balance between cholinergic and dopaminergic signaling in the basal ganglia resulting in the diminishing of tremor. Amantadine is indicated for alleviating levodopa-induced dyskinesia; however, its mechanism of action is now unknown.

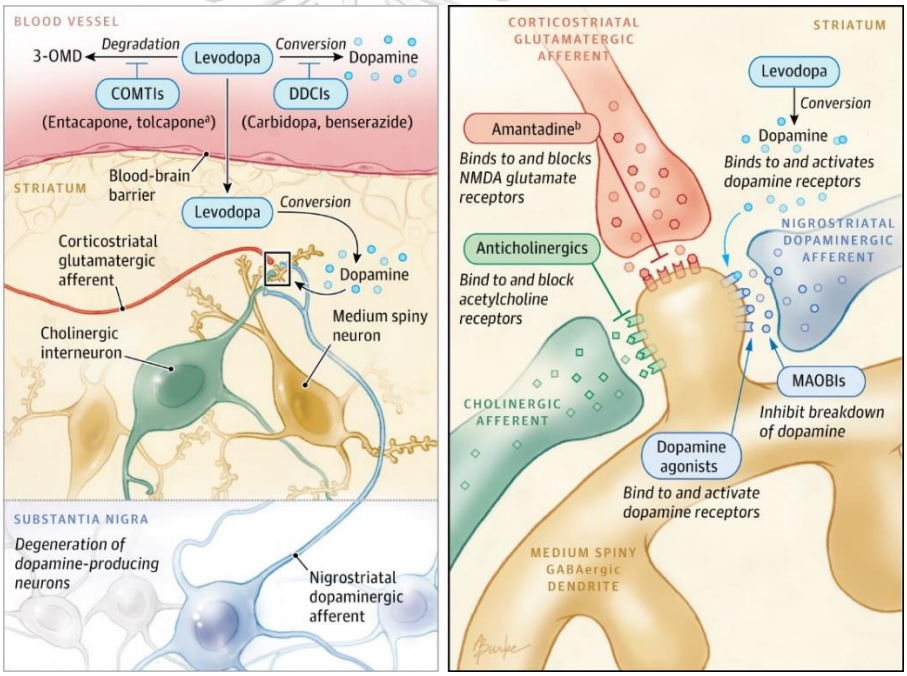


Figure 3 Mechanisms of action of drugs used in Parkinson's disease (Connolly & Lang, 2014)

Neuroprotective therapy is a novel strategy in the treatment of Parkinson's disease. The goal of neuroprotective therapy is to delay disease progression by protecting the neurons from degeneration. The

proposed neuroprotective mechanisms include anti-oxidant, mitochondrial protection, trophic factor improvement, anti-inflammation, and anti-apoptosis (Sarkar et al., 2016). Several neuroprotective candidates for Parkinson's disease have currently been studied such as coenzyme Q10, selegiline, rasagiline, resveratrol, ginsenoside, and curcumin **(Figure 4)** (Fu et al., 2015). Although the potential neuroprotective effects of these compounds have been reported in animal models and clinical trials (Fu et al., 2015), no treatment to date has been approved as a neuroprotective agent for Parkinson's disease.

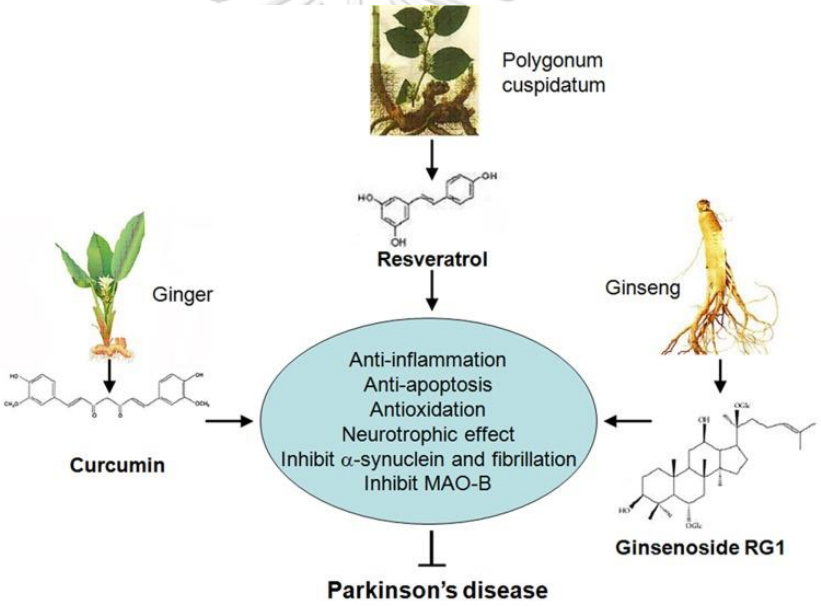


Figure 4 Natural compounds that exhibit neuroprotective effects in Parkinson's disease models (Fu et al., 2015).

2.2 Pathogenesis of Parkinson's disease

The main pathology of Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra and the reduction of dopamine levels in the striatum leading to motor and non-motor

symptoms (Fahn, 2003). The aggregations of **α**-synuclein proteins, called Lewy bodies, are also presented in the brain of Parkinson's disease patients (Guerrero et al., 2013). Overexpression of **α**-synuclein as well as the impairment of protein clearance system result in the aggregation of Lewy bodies and subsequently promote neuronal death (Le, 2014). Additionally, dopaminergic neuronal loss is caused by other molecular events including oxidative stress (Gaki & Papavassiliou, 2014), neuroinflammation (Wang et al., 2015) mitochondrial dysfunction and impaired calcium homeostasis (Rego & Oliveira, 2003) **(Figure 5)**. Genetic mutations in these molecular pathways found in familial Parkinson's disease help elucidate the molecular mechanisms of dopaminergic death.

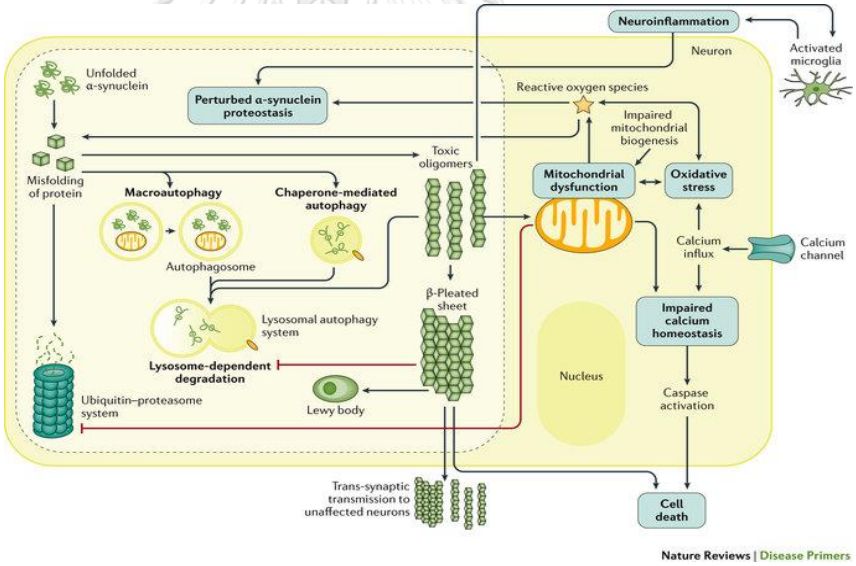


Figure 5 Major molecular pathways in the pathogenesis of Parkinson's *disease* (Poewe et al., 2017)

2.2.1 Genetic mutations

There is the increasing of evidence supported the roles of genetic factors in the pathogenesis of Parkinson's disease (Billingsley et al., 2018). Several gene mutations have been identified in familial Parkinson's

disease, including *SNCA, LRRK2, PARK2, PINK1*, and *DJ-1* **(Figure 6)**. *SNCA* mutation causes the overexpression of the **α**-synuclein protein, which leads to abnormal protein aggregation, Lewy body formation, and cellular oxidative stress (Klein & Schlossmacher, 2007). The leucine-rich repeat kinase-2 (*LRRK2*) gene encodes a protein called dardarin. This protein is a cytoplasmic kinase involving protein turnover and lysosomal degradation pathway (Schapira, 2006; Zimprich et al., 2004). Mutation of LRRK2 results in the abnormal protein clearance systems. Parkin, the protein encoded by the *PARK2* gene, plays a role in ubiquitination and protein degradation by proteasomes (Feng, 2006). Mutation of *PARK2* decreases protein clearance activity. Mutation of mitochondrial PTEN-induced putative kinase 1 gene (*PINK1*) causes mitochondrial dysfunction (Morais et al., 2014). *DJ-1* gene is associated with attenuation of cellular oxidative stress (Bonifati et al., 2003).

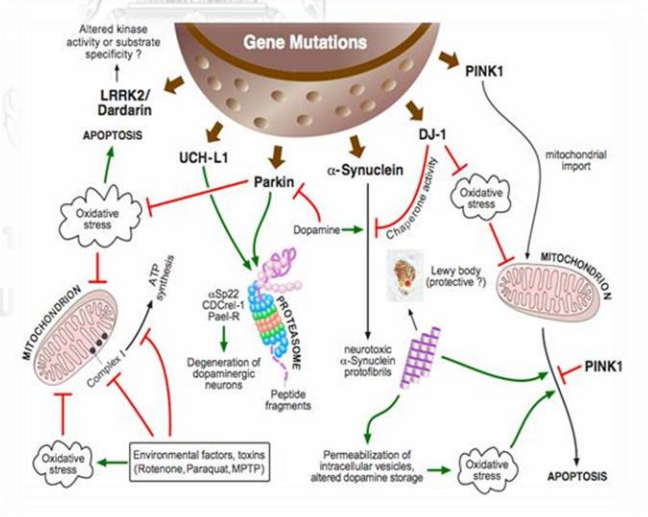


Figure 6 Mutations of Parkinson's associated genes (Poewe et al., 2017)

2.2.2 Protein aggregation and protein clearance

Protein aggregation has been proposed as one of the causes of cell death in Parkinson's disease. Lewy bodies, composed of **α**-synuclein protein, are aggregated in several brain areas of Parkinson's disease

patients such as basal ganglia, cortex, and limbic area (Gallegos et al., 2015).

An *in vitro* study revealed that **α**-synuclein interrupted cellular physiological processes including axonal transport, protein folding, protein clearance, mitochondria function, and immune response (Lashuel et al., 2002). Moreover, **α**-synuclein is a transmittable protein which can spread from an affected neuron to other neurons. Moreover, **α**-synuclein-transfected cells exhibited a marked increase in ROS production, leading to neuronal apoptosis (Xu et al., 2002). A single injection of **α**-synuclein into mouse's striatum caused cell-to-cell transmission leading to the loss of dopaminergic neurons in the substantia nigra and the impairment of motor coordination (Dehay et al., 2016; Luk et al., 2012a; Luk et al., 2012b).

Since aggregation of protein leads to neuronal death, neurons then activate their protein clearance systems, which are the ubiquitin-proteasome system and the autophagy-lysosomal pathway, as the defense mechanisms (Nedelsky et al., 2008) **(Figure 7)**. These systems maintain protein homeostasis by mediating the repair and removal of abnormal proteins (Dehay et al., 2013; Lim & Zhang, 2013). When the systems are inhibited by genetic mutations or neurotoxins, **α**-synuclein and its insoluble form can misfold, aggregate, accumulate inside the cells leading to apoptosis. Therefore, the prevention of protein aggregation is an important therapeutic strategy for Parkinson's disease (Jankovic, 2019).

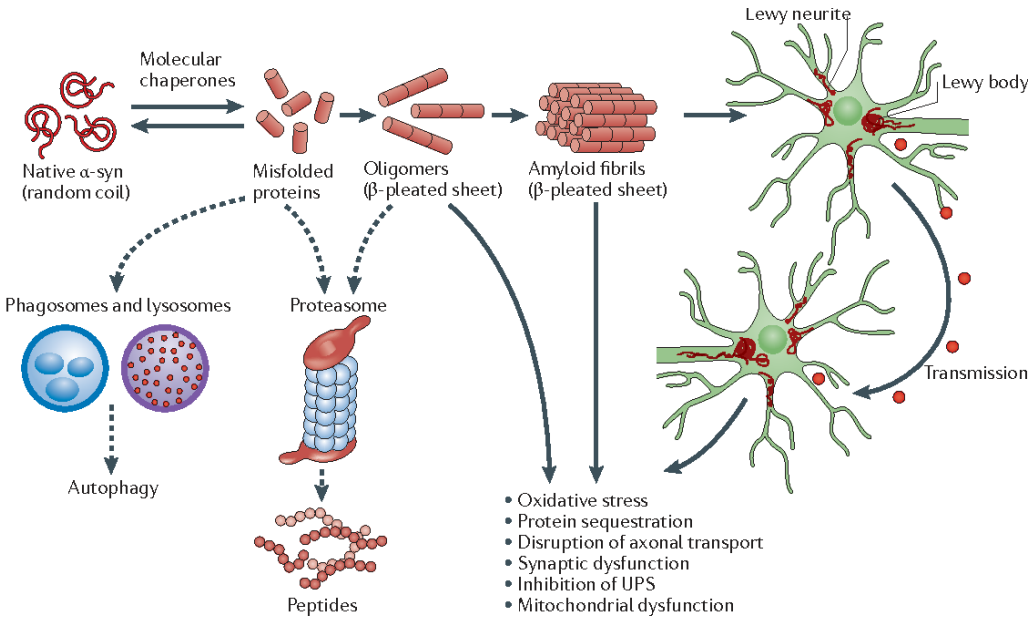


Figure 7 Protein aggregation and protein clearance systems (Irwin et al., 2013)

2.2.3 Neuroinflammation

In Parkinson's disease patients, the level of cytokines and inflammation markers are drastically elevated, suggesting that inflammation play roles in the pathogenesis of Parkinson's disease (Kim et al., 2018). The neuroinflammation in the brain is conducted in respondse to the aggregated α-synuclein (Hirsch & Hunot, 2009). These aggregated proteins then released into paracellular space and subsequently cause astrocyte and microglia activation, proinflammatory cytokine released, and paracellular damage. Cytokines, including interleukin-1, interleukin-6, and tumor necrosis factor (TNF-α) activate nitric oxide and superoxide production, contributing to oxidative stress (Hirsch & Hunot, 2009). Furthermore, activated microglia can degrade functional protein, cause mitochondria dysfunction, interrupt axonal transport and induce neuronal apoptosis (Qian et al., 2010; Sochocka et al., 2017) **(Figure 8)**.

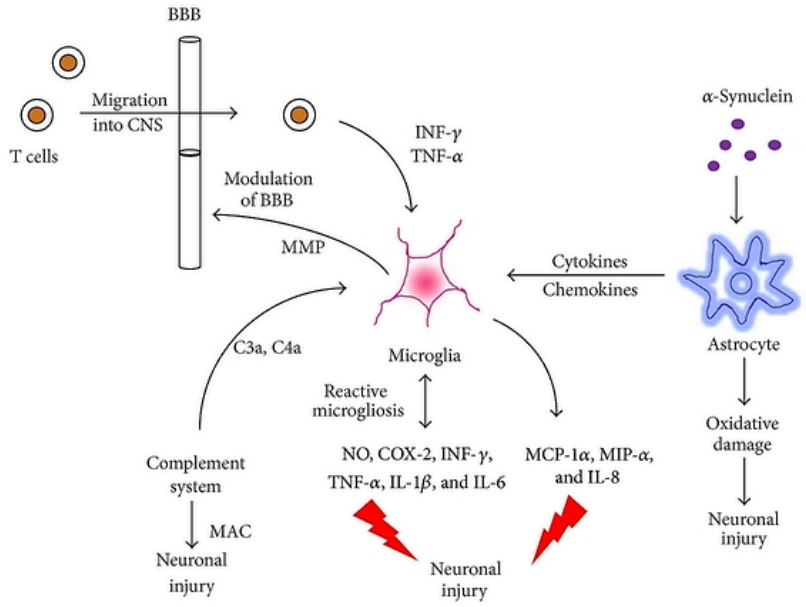


Figure 8 Molecular neuroinflammation in Parkinson's Disease (More et al., 2013)

2.2.4 Oxidative stress

Excessive reactive oxygen species (ROS) levels disturbs cellular homeostasis and causes neuronal apoptosis and neurodegeneration (Multhaup et al., 1997). A physiological level of ROS is mainly produced within mitochondria during electron transport chain (ETC). Mitochondrial dysfunction leads to abnormal ROS production (Park et al., 2018). Antioxidants and antioxidant enzymes, such as glutathione, superoxide dismutase (SOD) and catalase (CAT), regulate the excessive ROS levels (Lotharius & Brundin, 2002). However, the imbalance between antioxidant defenses, and ROS levels have been observed in Parkinson's disease (Gaki & Papavassiliou, 2014; Lotharius & Brundin, 2002) **(Figure 9)**. The oxidized DNA, lipids, and proteins were found largely in brain tissues of familial and sporadic Parkinson's disease patients (Bosco et al., 2006; Nakabeppu et al., 2007). Moreover, neurotoxin-induced animal models of

Parkinson's disease also demonstrates the increase of oxidative stress levels in brain tissues (Perier et al., 2003). Many antioxidants such as sesame, vitamin E and melatonin have shown to prevent oxidative stress and protect the nigrostriatal neurons in animal models of Parkinson's disease (Filograna et al., 2016; Sriram et al., 1997; Vila & Przedborski, 2003).

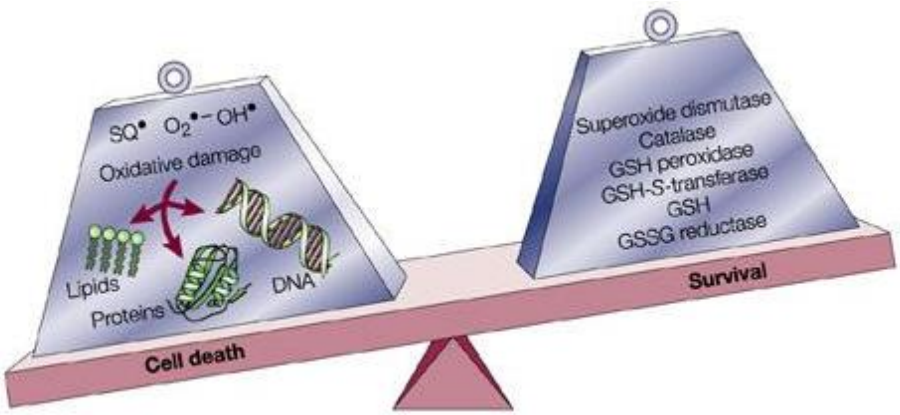


Figure 9 An imbalance between the ROS production antioxidant defense could contribute to the pathogenesis of Parkinson's disease (Lotharius & Brundin, 2002).

2.2.5 Mitochondrial dysfunction

Mitochondria are double membrane organelles which regulate cellular functions such as ATP synthesis, calcium homeostasis, and apoptosis induction (Winklhofer & Haass, 2010). The role of mitochondria in Parkinson's disease was unintentionally discovered as 1-methyl4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor, caused parkinsonism in abused patients (Przedborski et al., 2000). It has been later identified that the inhibition of mitochondrial complex I enzyme leads to Parkinsonian phenotypes (Halbach, 2005). Post-mortem studies revealed the reduction of mitochondria complex I

activity, mitochondrial membrane potential and ATP levels in Parkinson's disease patients (Schapira et al., 1990; Sharma et al., 2015). Importantly, administration of complex I inhibitors, such as MPTP and rotenone, can induce the selective loss of dopaminergic neurons and produce parkinsonism behaviors in animals (Halbach, 2005). The inhibition of complex I enzyme disturbs the flow of electron in electron transport chain leading to ROS production, oxidative stress, ATP depletion and neuronal cell death (Sherer et al., 2002; Zhang et al., 2001) **(Figure 10)**. As dopaminergic neurons are susceptible to oxidative stress and bioenergetics crisis (Haddad & Nakamura, 2015), the protection of mitochondria function is one of the promising approaches for neuroprotective strategy in Parkinson's disease.

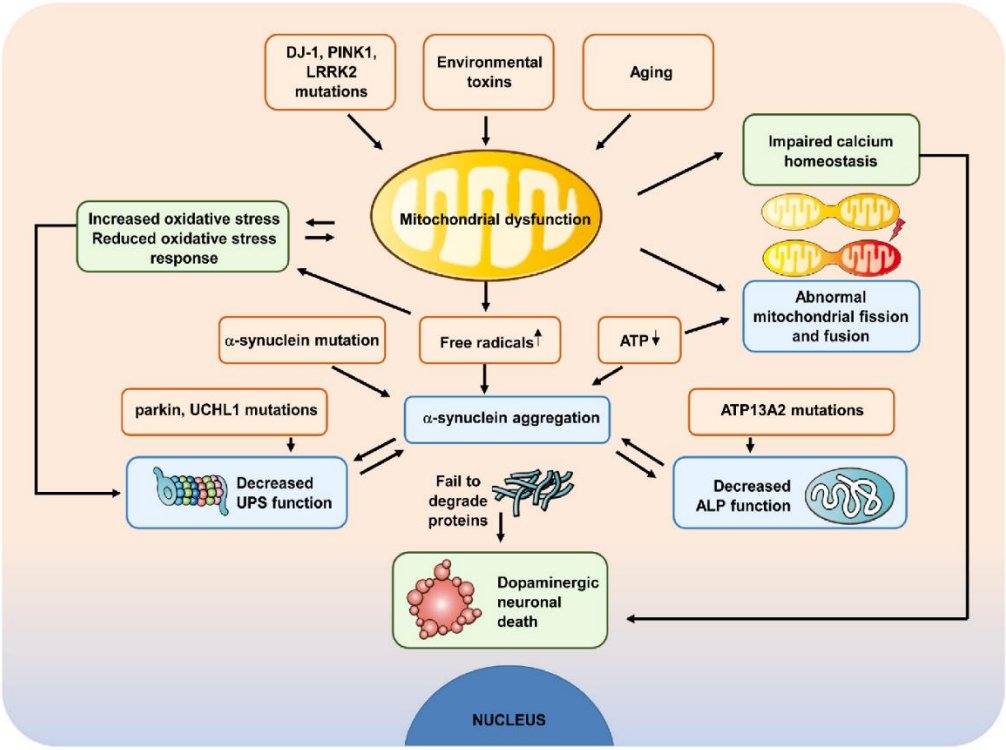


Figure 10 Mitochondrial dysfunction as a central role in the pathogenesis of Parkinson's disease (Moon & Paek, 2015)

2.2.6 Excitotoxicity

Excitotoxicity is a process of increased Ca^{2+} influx into the cytosol, causing physiological disruption and cell death (Ambrosi et al., 2014). Generally, regulations of Ca^{2+} influx rely on glutamate N-methyl-D-aspartate (NMDA) receptor. Activation of NMDA receptors causes Ca^{2+} influx and subsequently induces many physiological cascades and signaling transductions associated with memory and learning (Castellano et al., 2001). Overactivation of NMDA receptor occurs in Parkinson's disease (Dong et al., 2009), and consequently evokes oxidative stress, inflammation, mitochondrial dysfunction and neuronal apoptosis (Ambrosi et al., 2014) **(Figure 11).**

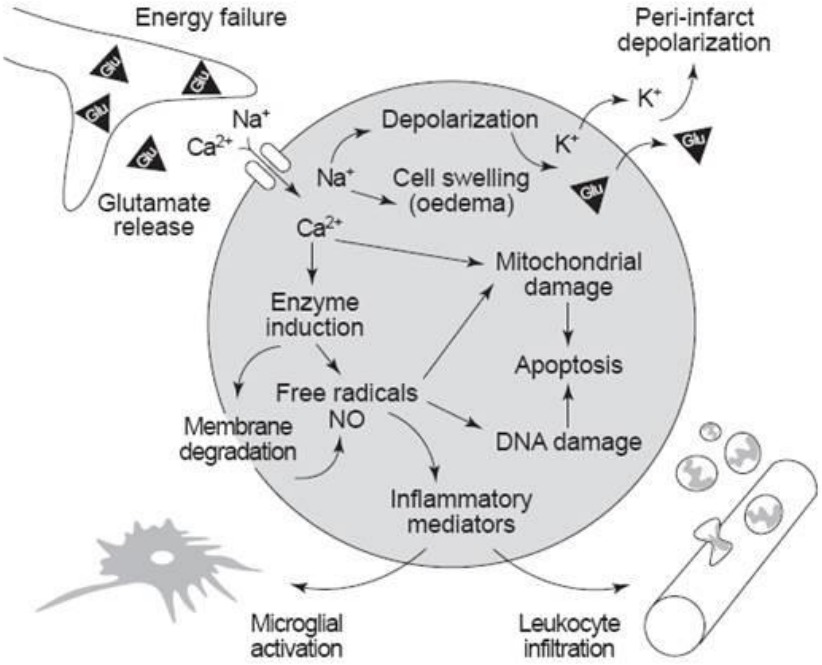


Figure 11 Molecular mechanisms of excitotoxicity in neurodegenerative disease (Dirnagl et al., 1999)

2.3 Autophagy and mitophagy

Autophagy is a cellular self-degradative program that plays the roles in removing aggregated proteins and damaged organelles (Anding & Baehrecke, 2017). In the mammalian cell, autophagy can be classified into macro-autophagy, microautophagy, and chaperone-mediated autophagy. Macro-autophagy mostly degrades aggregated proteins and damaged organelles by engulfing and transferring them to lysosome. Micro-autophagy removes small cytosolic components by transferring small cytosolic components directly to lysosome while chaperone-mediated autophagy transfers the misfolded proteins to the lysosome by chaperone proteins.

Macro-autophagy initiates by forming double membrane-bound vesicle called autophagosome. Beclin-1 is a platform for enabling the assembly of various proteins associated with autophagosome formation. Then, autophagy-related protein and the processing of LC3-phosphatidylethanolamine mediate the autophagosome elongation. Finally, autophagosome containing aggregated proteins and damaged organelles is fused with a lysosome to digest deficit proteins and release the recyclable substrates (Anding & Baehrecke, 2017) **(Figure 12)**.

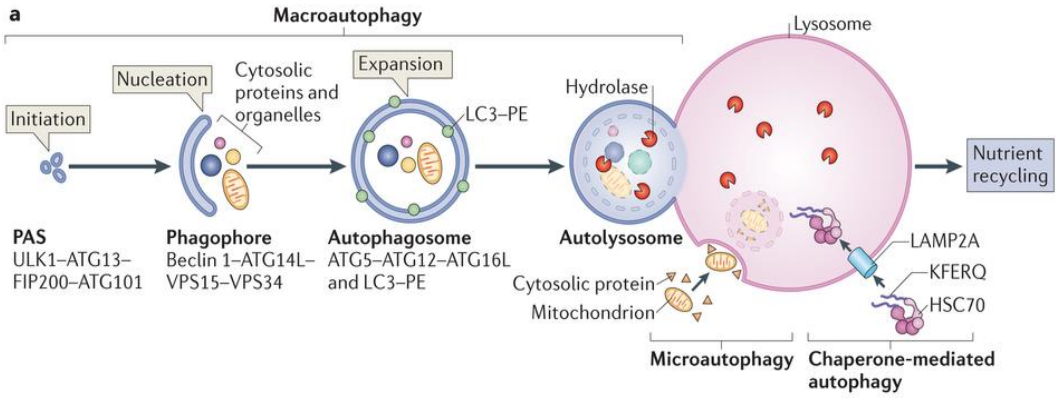


Figure 12 Autophagic-lysosomal system (Kaur & Debnath, 2015)

Mitophagy is a macro-autophagy process that selectively degrades damaged mitochondria (Martinez-Vicente, 2017). Since mitochondria dysfunction leads to molecular events of cell death, mitophagy helps maintain cellular homeostasis by clearance of the damaged mitochondria (Anding & Baehrecke, 2017; Rodriguez-Enriquez et al., 2006) **(Figure 13)**. In mitophagy, autophagosome engulfs the damaged mitochondria and further fused with a lysosome for digesting dysfunctional mitochondria. The digested substrates, including amino acid, are then released and reused for generating a new functional mitochondrion (Anding & Baehrecke, 2017). The depolarization of the mitochondrial membrane triggers mitophagy in response to hypoxia, chemical uncoupler, and oxidative stress (Anding & Baehrecke, 2017; Martinez-Vicente, 2017).

Mitophagy impairment occurs in the models of Parkinson's disease. For example, rotenone decreased the mitophagy process and its protein markers in SH-SY5Y and PC12 cells (Wu et al., 2015; Xiong et al., 2013; Yuan et al., 2015). In addition, rapamycin, an autophagic enhancer, attenuated dopaminergic neurodegeneration in MPTP-treated mice (Dehay et al., 2010). The neuroprotective actions of autophagy-enhancing agents such as metformin, curcumin, and resveratrol have been reported *in vivo* and *in vitro* models of Parkinson's disease (Moors et al., 2017). Thus, mitophagy enhancement is another promising strategy for Parkinson's disease management (East et al., 2014; Moors et al., 2017).

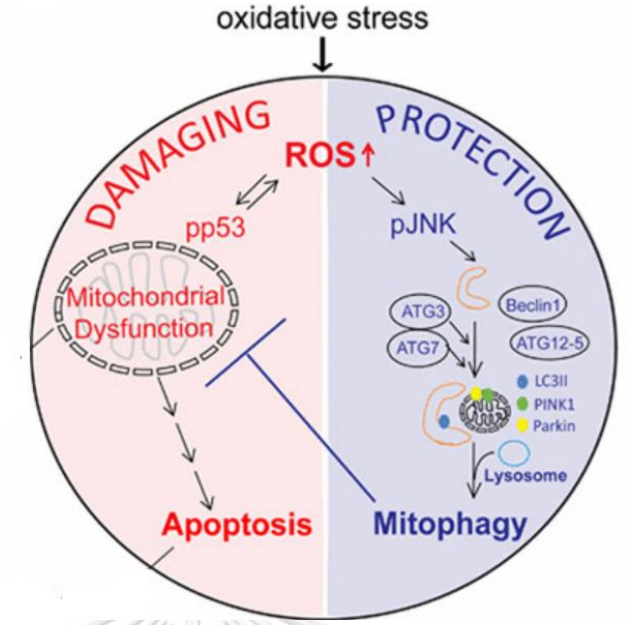


Figure 13 Mitophagy as a protective mechanism in cells (Lee et al., 2016)

2.4 Rotenone model of Parkinson's disease

As mitochondria dysfunction is the important pathology of Parkinson's disease, rotenone, a mitochondrial complex I inhibitor, has been used widely in the model of Parkinson's disease *in vivo* and *in vitro* (Perier et al., 2003). Rotenone, administered by subcutaneous, intravenous and intraperitoneal injections to the rats, can cause the selective loss of the nigrostriatal dopaminergic neurons, depletion of dopamine level, and α-synuclein aggregations leading to parkinsonism behaviors (Cannon et al., 2009; Perier et al., 2003; Sherer et al., 2003a; Testa et al., 2005; Tieu, 2011). Many behavioral tests including cylinder test, movement initiation test, open-field test, rotarod test, and catalepsy test have been used for detecting motor deficits in rotenone models. It was shown that rotenone administration decreased locomotor activity, prolonged movement latency and reduced rotarod performance (Fleming et al., 2004; Inden et al., 2007; Sharma & Nehru, 2013; Sherer et al., 2003b) The motor deficits induced by rotenone are dopamine-dependent

as the administration of a dopamine agonist, apomorphine, can improve the motor symptoms (Cannon et al., 2009).

Rotenone has been used to induce cytotoxicity in many dopaminergic-liked cell lines such as PC12, SH-SY5Y, and LUHMES cells (Nataraj et al., 2017; Sai et al., 2008; Smirnova et al., 2016). Molecular mechanisms of rotenone-induced cytotoxicity in PC12 cell consisted of a decrease of cellular ATP level, an increase of ROS production, and the impairment of mitochondrial membrane potential (Marella, 2007). In addition, rotenone impaired autophagy-lysosomal pathway, and subsequently caused α-synuclein accumulation and apoptosis in PC12 and SH-SY5Y cells (Wu et al., 2015; Xiong et al., 2013; Yuan et al., 2015). Taken together, the rotenone model is a good model to recapitulate the pathologies and molecular mechanisms of Parkinson's disease *in vitro* and *in vivo*.

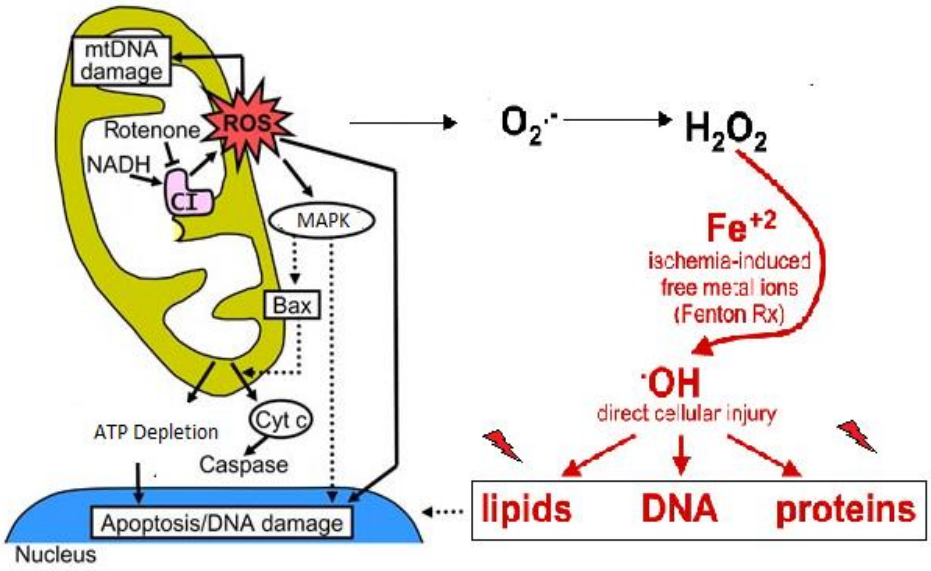


Figure 14 Mechanisms of action of rotenone toxicity (Marella, 2007)

2.5 The standardized extract of *Centella asiatica* ECa233

Centella asiatica (Apiaceaea) has been used in Chinese, Ayurvedic and Thai traditional medicines for the treatment of wound, varicose vein and eczema (Gohil et al., 2010; Vaidya, 1997). *Centella asiatica* has been approved to use externally as a wound healing agent according to the National list of essential medicines of Thailand (Thai-FDA, 2012). Pharmacological effects of *Centella asiatica* include wound healing effect (Jin et al., 2015), anxiolytic effect (Wijeweera et al., 2006), learning and memory improvement (Rao et al., 2005; Veerendra Kumar & Gupta, 2002), anti-inflammation (Sharma et al., 2014), anti-oxidation (Anand et al., 2010) and neuroprotective effects (Kumar & Gupta, 2003).

The major bioactive constituents of *Centella asiatica* are asiatic acid, asiaticoside, madecassic acid, and madecassoside (Inamdar et al., 1996) **(Figure 15)**. Previous studies demonstrated many pharmacological effects of madecassoside, madecassic acid, asiaticoside, and asiatic acid including neuroprotective effect, anti-inflammation, neuritogenic effect and antioxidative effect (Ling et al., 2017; Xiong et al., 2009; Xu et al., 2013; Xu et al., 2012)

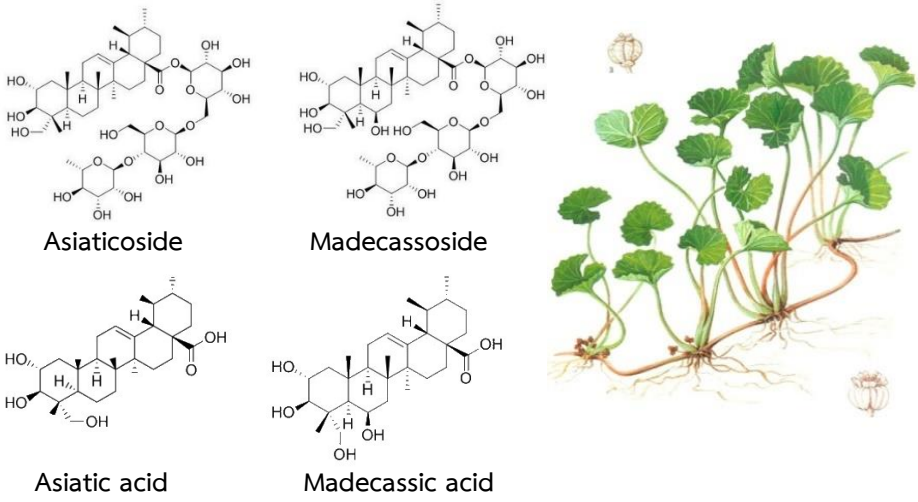


Figure 15 *Centella asiatica* and structure of the major bioactive constituents (Anukunwithaya et al., 2017b)

Natural extracts generally vary in their compositions leading to unsteady biological responses. To avoid that problem, Faculty of Pharmaceutical Sciences, Chulalongkorn University have developed a standardized extract of *Centella asiatica* ECa233. ECa233 are a white to an off-white powder containing triterpenoid glycosides not less than 80%. The ratio between madecassoside and asiaticoside are maintained at 1.5:1 (Saifah E, 2009).

2.5.1 Pharmacokinetic studies of ECa233

A single-dose of ECa233 caused a rapid absorption of madecassoside and asiaticoside. T_{max} of madacassoside and asiaticoside were 0.22 and 0.62 hours, respectively (Hengjumrut et al., 2017). C_{max} of madecassoside and asiaticoside were demonstrated to be dose-dependent after oral administration of ECa233 in rats. Within 1 hour after ECa233 administration, madecassoside and asiaticoside accumulated in brain, stomach, kidney, liver, and skin (Anukunwithaya et al., 2017b). ECa233 did not affect of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A activities at the dose up to 2000 mg/kg (Kulthong et al., 2009). Madecassoside and asiaticoside were mainly excreted as their acid forms including madecassic acid and asiatic acid suggesting the extensive metabolism of madecassoside and asiaticoside (Anukunwithaya et al., 2017b; Hengjumrut et al., 2017).

Furthermore, administration of ECa233 increased AUC and elimination half-life of madecassoside and asiaticoside compared to those of pure compounds administered alone (Hengjumrut et al., 2017). This result indicated a superior pharmacokinetic profile of ECa233 which might provide a better biological activity of ECa23. The same study also

reported the interconversion between asiaticoside and madecassoside that might account for the increased AUC and elimination half-life of madecassoside and asiaticoside after ECa233 administration (Hengjumrut et al., 2017).

2.5.2 Safety and toxicity test

ECa233 had a good safety profile in animal studies (Chivapat et al., 2011). Acute toxicity study in mice found that oral administration of ECa233 up to 10 g/kg caused neither toxic sign nor death within 14 days. Moreover, the sub-chronic study showed no significant change in body weight, food consumption, and health after giving the rats with ECa233 10, 100, and 1,000 mg/kg/day. All ECa233-treated rats had no pathological lesion of the internal organs. Therefore, it can be concluded that ECa233 has no acute toxicity in the dose up to 10 g/kg as well as sub-chronic toxicity up to 1,000 mg/kg (Chivapat et al., 2011). In addition, acute and sub-chronic administration of ECa233 did not cause toxicity in rats, suggesting ECa233 is a promising candidate in drug development (Chivapat et al., 2011).

2.5.3 Pharmacological activities of ECa233

The effect of ECa233 in CNS has been increasingly investigated. It was shown that normal rats receiving 30 mg/kg of ECa233 for 14 days had higher cognitive performance than untreated rats suggesting its learning and memory enhancing activity (Wattanatorn et al., 2008). Anxiolytic activity of ECa233 was also observed in acutely, and chronically stressed mice (Wanasuntronwong et al., 2012). ECa233 (10 mg/kg) protected against neurotoxicity induced by β-amyloid (Kam-eg et al., 2009). In addition, ECa233 (10 and 30 mg/kg) improved learning and memory

deficits induced by common carotid occlusion in mice (Tantisira et al., 2010). *In vitro* study also revealed neuritogenic effect of ECa233 (1-100 μg/mL) in IMR-32 neuroblastoma cell (Wanakhachornkrai et al., 2013). Although ECa233 exerts various pharmacological effects in CNS, the neuroprotective effect of ECa233 in Parkinson's disease model is not yet clarified. Therefore, this study aims to demonstrate the neuroprotective effects of Eca233 against rotenone-induced Parkinson's disease model.

CHAPTER 3
MATERIALS AND METHODS

3.1 Material

3.1.1 Chemicals and Reagents

1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St. Louis, MO. USA)

2′-7′-dichlorodihydrofluorescein diacetate (Thermo Fisher Scientific, USA)

3, 30-diaminobenzidine (Wako, Japan)

Absolute ethanol (Merck, Darmstadt, FR. Germany)

Acetic acid glacial (BDH Laboratory Supplies, Poole, Dorset, England)

Apomorphine (Sigma-Aldrich, St. Louis, MO. USA)

Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO. USA)

Carboxymethyl cellulose CMC (Sigma-Aldrich, St. Louis, MO. USA)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO. USA)

Dulbecco's Modified Eagle Medium: F-12 (Thermo Fisher Scientific, USA)

Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, USA)

GlutaMAX (Thermo Fisher Scientific, USA)

N-butanol (Sigma-Aldrich, St. Louis, MO. USA)

Nonidet P40 (Sigma-Aldrich, St. Louis, MO. USA)

Paraformaldehyde (Sigma-Aldrich, St. Louis, MO. USA)

Penicillin-streptomycin (Thermo Fisher Scientific, USA)

Pentobarbital sodium (Nembutal®, CEVA Sante' animale, Belgium)

Phenylmethylsulfonyl fluoride

Potassium phosphate dibasic (Sigma-Aldrich, St. Louis, MO. USA)

Potassium phosphate monobasic (Sigma-Aldrich, St. Louis, MO. USA)

Pyridine (Merck, Darmstadt, FR. Germany)

Resazurin powder (Sigma-Aldrich, St. Louis, MO. USA)

Rotenone (Sigma-Aldrich, St. Louis, MO. USA)

Skim milk (Sigma-Aldrich, St. Louis, MO. USA)

Sodium carbonate (Fisher Scientific UK Limited, Leicestershire, UK)

Sodium chloride (Sigma-Aldrich, St. Louis, MO. USA)

Sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO. USA)

Sodium fluoride (Sigma-Aldrich, St. Louis, MO. USA)

Sodium hydroxide (Merck, Darmstadt, FR. Germany)

Sodium orthovanadate (Sigma-Aldrich, St. Louis, MO. USA)

Sucrose (Merck, Darmstadt, FR. Germany)

Sunflower oil (Morakot®, Thailand)

Tetramethylrhodamine, ethyl ester (Thermo Fisher Scientific, USA)

Thiobarbituric acid (Sigma-Aldrich, St. Louis, MO. USA)

Tris (Sigma-Aldrich, St. Louis, MO. USA)

Tris-HCl (Sigma-Aldrich, St. Louis, MO. USA)

Tween-20 (Sigma-Aldrich, St. Louis, MO. USA)

Xylene (Sigma-Aldrich, St. Louis, MO. USA)

3.1.2 Equipment

CG842 laboratory pH meter (SCHOTT® Instruments GmbH, Germany)

Cryostat microtome (LEICA, Germany)

Dounce homogenizer (Glas-Col®, USA)

Himac Refrigerated centrifuge machine (Hitachi, Ltd., Japan)

IX51® Inverted Microscope (Olympus America Inc., USA)

Microplate reader (CLARIOstar®,BMG LABTECH, Germany)

Mikro 20 centrifuge machine (Hettich, Switzerland)

PG 2002-S Balance (Mettler Toledo, Columbus, OH. USA)

Power supply (PowerPac,USA)

TE124S Analytical balance (Sartorius AG., Germany)

Temperature controlled water bath (Memmert, Germany)

Trans-Blot SD semi-dry electrophoretic transfer (Trans-Blot,USA)

VideoMOT2 system (TSE Systems GmbH, Bad Homburg, Germany)

VM300 Vortex mixer (Axiom®, Germany)

3.1.3 Test compounds preparation

ECa233 was provided by Nature Idea Company Limited, Bangkok, Thailand. ECa233 was freshly prepared by suspending in 0.5% carboxymethyl cellulose (CMC) to obtain the desired concentrations (10, 30 and 100 mg/mL).

Rotenone's stock solution was prepared by dissolving 150 mg of rotenone in 1 mL of 100% DMSO and stored in the -20ºC refrigerator until the use. The working solution was freshly prepared by diluting stock solution in sunflower oil to obtain 2.5 mg/mL of rotenone.

Apomorphine was dissolved in sterile water at the concentration of 0.5 mg/mL.

3.2 Methods

3.2.1 *In vivo* study

3.2.1.1 Animals

Eight-week old adult male Wistar rats (300-350 g) were purchased from the National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand. Rats were acclimatized and housed in polypropylene cages in a group of two. The room conditions were adjusted to 24 ± 2° C with 60 ± 10% humidity and 12 light/dark cycles. Food and water were provided ad libitum. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (Approval no. 15-33-004).

3.2.1.2 Experimental design

Rats were randomly assigned to five experimental groups (n=10/group) including control, rotenone-induced parkinsonism (PD) and PD pre-treated with ECa233 either 10, 30 or 100 mg/kg (PD-ECa10, PD-ECa30, and PD-ECa100, respectively) **(Figure 16)**.

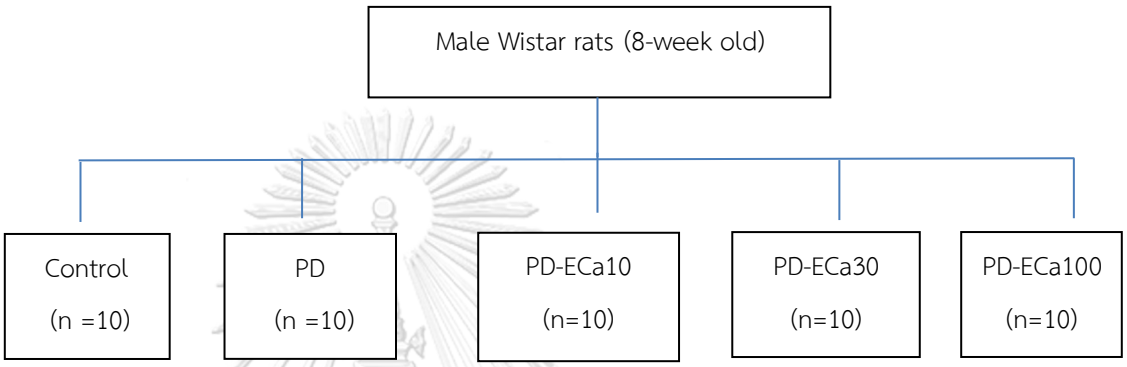


Figure 16 The experimental groups

Rats in control and PD groups received 0.5% CMC (1 mL/kg, p.o.) while rats in PD-ECa10, PD-ECa30 and PD-ECa100 received ECa233 10, 30 and 100 mg/kg p.o., respectively for the first 14 days. On day 15 – 20, rats in control group received 0.5% CMC (1 mL/kg, p.o.) followed by 2% DMSO in sunflower oil (1 mL/kg, i.p.) while rats in PD group received 0.5% CMC (1 mL/kg, p.o.) followed by rotenone (2.5 mg/kg, i.p.). Rats in PD-ECa10, PD-ECa30, and PD-ECa100 were given the same dose of ECa233 followed by rotenone (2.5 mg/kg, i.p.) throughout day 15-20. Locomotor activity was performed on day 1 (before treatment), 14, 17, and 20. On day 21, the apomorphine challenge was then conducted. On day 22, all rats were euthanized using pentobarbital (100 mg/kg, i.p.) **(Table 1)**. Five rats per group were transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA) for fixation. Brains were removed and stored in 4% PFA for further histological investigation. Brains of the remaining rats (n=5/group) were rapidly removed and cut midsagittally. The left brains

were freshly prepared for mitochondria fraction to measure mitochondrial complex I activity. The right brains were kept in -80°C for later biochemical analysis.

Table 1 experimental plan and drug administration.

Group	Experimental day																				
	Pre-treatment														Rotenone						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Control	0.5% CMC (p.o.)														0.5% CMC (p.o.) + 2% DMSO (i.p.)						Apomorphine challenge	Brain collection
PD	0.5% CMC (p.o.)														0.5% CMC (p.o.) + 2.5 mg/kg rotenone (i.p.)							
PD-ECa	ECa233 10, 30 or 100 mg/kg (p.o.)														ECa233 10, 30 or 100 mg/kg (p.o.) + 2.5 mg/kg rotenone (i.p.)							

3.2.1.3 Open field test

To evaluate the locomotor activity, each rat was placed in a black square box (50×50×40 cm.) for 5 minutes. The distance, ambulation time, and immobilization time were recorded and analyzed using VideoMOT2 system.

3.2.1.4 Apomorphine challenge

To determine whether the deteriorated locomotor activity was a dopamine-dependent, apomorphine (0.5 mg/kg), a dopamine agonist, was subcutaneously injected to all rats on day 21. Five minutes later, rats were performed the open field tests. Data were then collected and analyzed.

3.2.1.5 Immunohistochemical analysis

Tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine synthesis, was used as a histological marker of dopaminergic neurons in this study. The histological staining technique was modified from Park et al. (Park et al., 2015). Briefly, brains in 4% PFA were transferred into 30% sucrose and further incubated for 48 hours. Next, brains were coronally sectioned (40 µm) using a cryostat at -20 °C. Sections at +2.16, +2.04, and +1.92 from bregma and -5.04, -5.20 and -5.28 from bregma were selected to represent the striatum and substantia nigra respectively (Paxinos & Watson, 2007). The selected sections were incubated in 0.1% H_2O_2 for 30 minutes and transferred to 5% skim milk for 1 hour. The sections were subsequently incubated with primary anti-TH antibody (1:1000, Merck Millipore, Germany) at 4°C overnight followed by a 2-hour incubation with secondary biotin-conjugated anti-goat antibody (1:500, Merck Millipore, Germany) at room temperature. The antibody-antigen complexes were trapped with Avidin-Biotin Complex (Vector, CA, USA) followed by reacting with 3, 30-diaminobenzidine (Wako, Japan). Each section was later placed onto a glass slide and air-dry for 12 hours. For dehydration, each slide was immersed in a serial concentration of ethanol from 50%,

70%, 95%, 100% and finally in xylene. To preserve the samples, each slide was mounted using mounting media and closed with a cover slid.

To quantify the dopaminergic neurons in the substantia nigra, total TH-positive neurons were counted individually for each rat. For the striatum, the intensity of TH-positive neurons was analyzed by converting the images to gray scale, and measured the gray levels using Image J software. The values were subtracted with gray levels of the corpus callosum to obtain background correction.

3.2.1.6 Mitochondrial complex I activity assay

Complex I enzyme activity was measured using Complex I enzyme activity assay kits (Abcam, Cambridge, UK). Brains were homogenized in 0.1 M ice-cold PBS (1:10 W/V) using Dounce homogenizer. Homogenates were extracted mitochondrial fraction by adding 10 µL of detergent provided in the kit to 90 µL of homogenate and incubated for 30 minutes. Homogenates were centrifuged at 16,000 g for 20 minutes. Supernatants (200 µL) were loaded into ELISA plate and incubated for 3 hours. After incubation, samples were removed, followed by adding 300-µL assay solutions into each well. Mitochondria complex I activity was measured using a microplate reader (kinetic mode: OD 450 nm for 30 minutes). The activity of the enzyme was normalized by total protein using the Bradford protein quantification kit (Abcam, Cambridge, UK).

3.2.1.7 Thiobarbituric acid reactive species (TBARS) method

Lipid peroxidation was determined by measuring malondialdehyde (MDA). Brains were weighed and added 1:10 w/v of 0.1 M PBS at 4°C. Samples were then homogenized and centrifuged at 2,000 g for 15 minutes. After that, 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid, and 0.2 mL of 8.1% sodium dodecyl sulfate (SDS)

were mixed with 100 µL of the samples. The mixtures were heated at 95°C for 60 minutes. Five-mL of N-butanol: pyridine solution (15:1 v/v) was then added and centrifuged at 4000 rpm for 10 minutes. The supernatants were collected and measured OD at 532 nm using a microplate reader (SpectraMax Plus 384, CA, USA)

3.2.1.8 Protein expression

Brain pellet was lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and sodium fluoride). Homogenates were centrifuged at 12000 g for 20 minutes. Supernatants were collected and measure total protein concentration using BCA protein assay kit (Thermo Fisher Scientific, USA). Total protein concentration was adjusted to 100 µg using loading buffered. Samples were boiled at 95°C for 5 minutes then electrophoresed in 12% SDS-PAGE at 60 V for 2.5 hours. Proteins were further electrotransferred onto a PVDF membrane at 9 V for 1 hour and unspecific proteins were blocked using 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 hour. The membrane was then incubated at 4°C overnight with the primary antibodies against catalase enzyme (1:500, Santa Cruz, USA), SOD enzyme (1:500, Santa Cruz, USA) and GAPDH (1:1000, Merck Millipore, Germany). Next, the membrane was triplicately washed with TBST and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:1000, Merck Millipore, Germany) at room temperature for 2 hours. The membrane was washed by TBST triplicately and visualized using the chemiluminescence detection reagents (Thermo Fisher Scientific, USA).

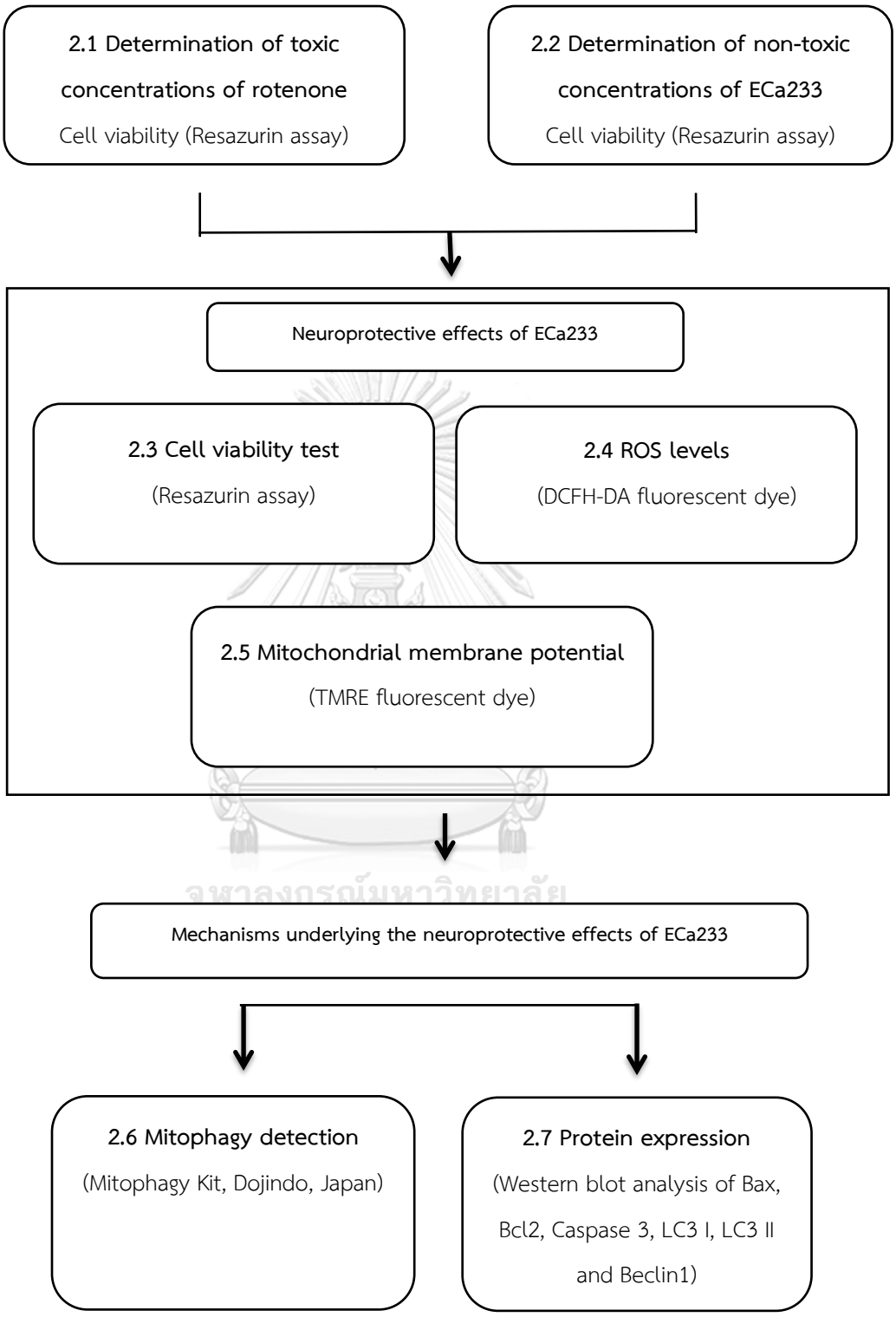


Figure 17 Experimental design for *in vitro* study

3.2.2 *In vitro* study

Human neuroblastoma SH-SY5Y cells were purchased from American type culture collection (ATCC, USA). Cells were cultured and maintained in DMEM/F12 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO_2 incubator at 37°C. Media were changed every two days, and subcultured (1:3) every five days.

SH-SY5Y cell is a neuroblast cell widely used in Parkinson's disease research. The cell is a subclone of the SK-N-SH cell line that was isolated from the bone marrow of a female neuroblastoma patient. SH-SY5Y has been reported to synthesize, release, take up, and store catecholamines, including dopamine (Filograna et al., 2015; McMillan et al., 2007). This cell also expressed tyrosine hydroxylase enzyme and dopamine transporter as markers of the dopaminergic neuron (Korecka et al., 2013; McMillan et al., 2007; Nisar et al., 2015). Thus, SH-SY5Y has been extensively used as a dopaminergic cell model to study various effects of Parkinson toxins on dopamine neurons *in vitro* (Arun et al., 2008; Chen et al., 2005; Huiyi Jiang, 2019).

To investigate the molecular mechanisms of ECa233, SH-SY5Y cells were treated with ECa233 or rotenone for cytotoxic screening. Non-toxic concentration of ECa233 and toxic concentration of rotenone were used to determine cell viability, ROS levels and mitochondrial membrane potential using resazurin assay, dichloro-dihydro-fluorescein diacetate (DCFH-DA) technique and tetramethylrhodamine, ethyl ester (TMRE) assay, respectively. To elucidate the molecular mechanisms of ECa233, mitophagy process and protein expression were measured using mitophagy detection and western blot analysis, respectively **(Figure 17)**.

3.2.2.1 Determination of toxic concentrations of rotenone

SH-SY5Y cells were seeded in 96-well plates at a density of 5 × 10^4 cells/200 µL. Cells were treated with rotenone at 50, 100, 250, 500, and 1000 nM for 48 hours (Nianyu Li). Cell viability was then determined using the resazurin assay. Briefly, ten-microliter of resazurin (0.1 mg/mL) was added into each well 4 hours prior to the end of the incubation time. The fluorescent intensity was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The percentage of cell viability was calculated as follows:

% cell viability = (Abs. sample/Abs. control) x100

3.2.2.2 Determination of non-toxic concentrations of ECa233

SH-SY5Y cells were seeded in 96-well plate at a density of 5 × 10^4 cells/200 µL. Cells were treated with ECa233 (5, 10, 25, 50, and 100 µg/mL) for 12 hours. Cell viability was then measured using resazurin assay.

3.2.2.3 Determination of neuroprotective effects of ECa233

SH-SY5Y cells were seeded in 96-well plates at a density of 5 × 10^4 cells/200 µL. Cells were pretreated with ECa233 (5, 10, 25, 50 and 100 µg/mL) for 12 hours followed by rotenone incubation for 48 hours. Cells viability was then measured using resazurin assay.

3.2.2.4 Determination of intracellular ROS levels

Cells were seeded in 96-well plates at a density of 5 × 10^4 cells/200 µL. Cells were incubated with ECa233 for 12 hours followed by rotenone either 6, 24 or 48 hours. 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) 25 nM was added at the end of rotenone incubation timepoints and incubated product (DCF) was quantified using microplate

reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

3.2.2.5 Determination of mitochondrial membrane potential

SH-SY5Y cells were seeded at a density of 5 × 10^4 cells/200 µL. After incubation with ECa233 for 12 hours followed by rotenone treatment for 48 hours, tetramethylrhodamine ethyl ester (TMRE) solution (150 nM) was added and incubated for 30 minutes. Fluorescent intensity was determined by a microplate reader (SpectraMax Plus 384, CA, USA) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Mitochondrial membrane potential ($\Delta\Psi$) was assessed by cationic TMRE fluorescent dye that selectively binds to living mitochondria membrane. The depolarized mitochondrial membrane fails to accumulate this fluorescent dye resulting in a weaker fluorescent intensity.

3.2.2.6 Mitophagy detection

SH-SY5Y cells were seeded in 96-well plates at a density of 5 x 10^5 cells/200 µL. After incubation with ECa233 for 12 hours, cells were incubated with 100 nmol Mitophagy Dye (Dojindo, Japan) at 37°C for 30 minutes. After incubation, media were removed, and cells were treated with rotenone for 48 hours. Principally, Mitophagy Dye is firstly accumulated in the mitochondria. The strong fluorescent intensity will be presented after mitochondria fuse to lysosome representing the mitophagy process **(Figure 18)**. Mitotophagy was then detected by a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

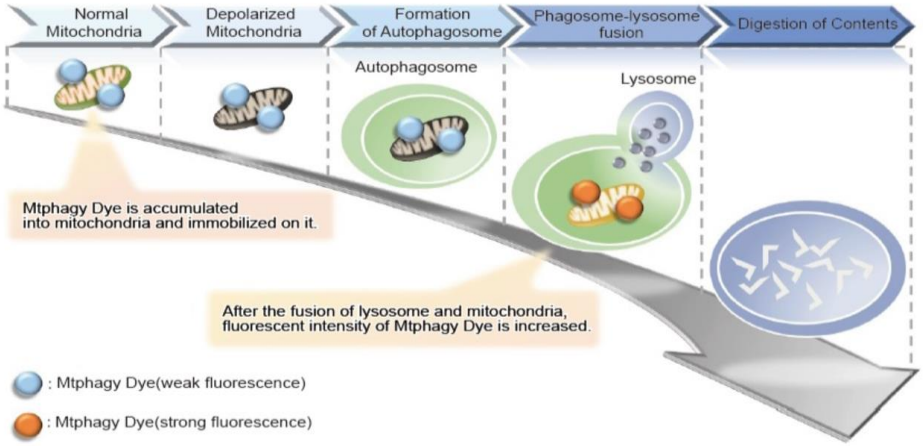


Figure 18 Principle of mitophagy detection (Dojindo, 2016)

3.2.2.7 Protein expression

SH-SY5Y cells were seeded in 6-well plates at a density of 2 x 10^6 cells/2 mL. After incubation with ECa233 for 12 hours followed by rotenone for 24 hours (Kang et al., 2017; Xiong et al., 2013), cells were collected for further analysis of autophagy and apoptosis-associated proteins using western blot analysis. The primary antibodies used in this study included Bax (1:1000, Cell signaling technology, USA), Bcl2 (1:1000, Sigma-Aldrich, USA) and caspase 3 (1:1000, Cell signaling technology, USA) for apoptosis pathway and LC3l/II (1:1000, Cell signaling technology, USA), and Beclin-1(1:1000, Cell signaling technology, USA) for mitophagy pathway and GAPDH (1:1000, Merck Millipore, Germany). The level of GAPDH protein was used for normalization of protein loading. The band density was measured using Image J software.

3.3 Statistical analysis

For *in vivo* study, results were expressed as mean ± S.E.M. of experimental data obtained from 5 – 10 rats. Differences among means were tested using one-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test. The results on the experimental day 21

(apomorphine challenge) were compared and analyzed with the results on an experimental day 20 using the dependent t-test. The mean differences were considered to be statistical significance when the P value was lesser than 0.05.

For *in vitro* study, three independent experiments were conducted. The results were expressed as mean ± S.E.M. and were analyzed using one-way ANOVA followed by Tukey's test or Fisher's LSD post-hoc test. The mean differences were considered to be statistical significance when the P value was lesser than 0.05.

CHAPTER 4

RESULTS

4.1 In vivo study

4.1.1 Locomotor activity

4.1.1.1 Total distances

The distances of rats were measured on experimental day 1, 14, 17, and 20**.** On day 1 as a baseline, the locomotor activity of rats was not different among the experimental groups. The mean distances of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups on day 1 were 2,356 ± 127.9, 2,324 ± 132, 2,485 ± 136.1, 2,208 ± 129.9 and 2,384 ± 153.6 cm, respectively ($F (4, 45) = 0.5399$, $p = 0.7072$, one-way ANOVA). The average distances of rats were also not significantly different on day 14, suggesting no motor-stimulating effect of ECa233 ($F (4, 45) = 0.6139$, $p = 0.6548$, one-way ANOVA). The average distances of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups on day 14 were 2297 ± 96.71, 2160 ± 138.8, 2264 ± 68.41, 2065 ± 72.49 and 2146 ± 182 cm, respectively. On day 17, the average distances of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 2320 ± 151.2, 1058 ± 153.6, 833.2 ± 116.1, 1259 ± 210.3 and 1178 ± 171.9 cm, respectively. One-way ANOVA revealed a significant effect of treatment on rat's distances ($F (4, 45) = 12.42$, $p<0.0001$) which rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups showed a significant decrease of mean distances compared to control ($p<0.001$, Fisher's LSD post-hoc test). On day 20, the average distances of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 2203 ± 150.8, 768.2 ± 64.1, 835.7± 94.79, 1278 ± 153.3 and 917.3 ± 126.3 cm, respectively. One-way ANOVA revealed a significant difference among experimental groups ($F (4, 45) = 23.44$, $p<0.0001$). Rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups

showed a significant decrease of mean distances compared to control ($p<0.0001$, Fisher's LSD post-hoc test). However, the average distances of PD-ECa30 rats were significantly higher than those of PD, PD-ECa10 and PD-ECa100 rats ($p<0.01$, $p<0.05$, $p<0.05$, respectively, Fisher's LSD post-hoc test) **(Figure 19)**.

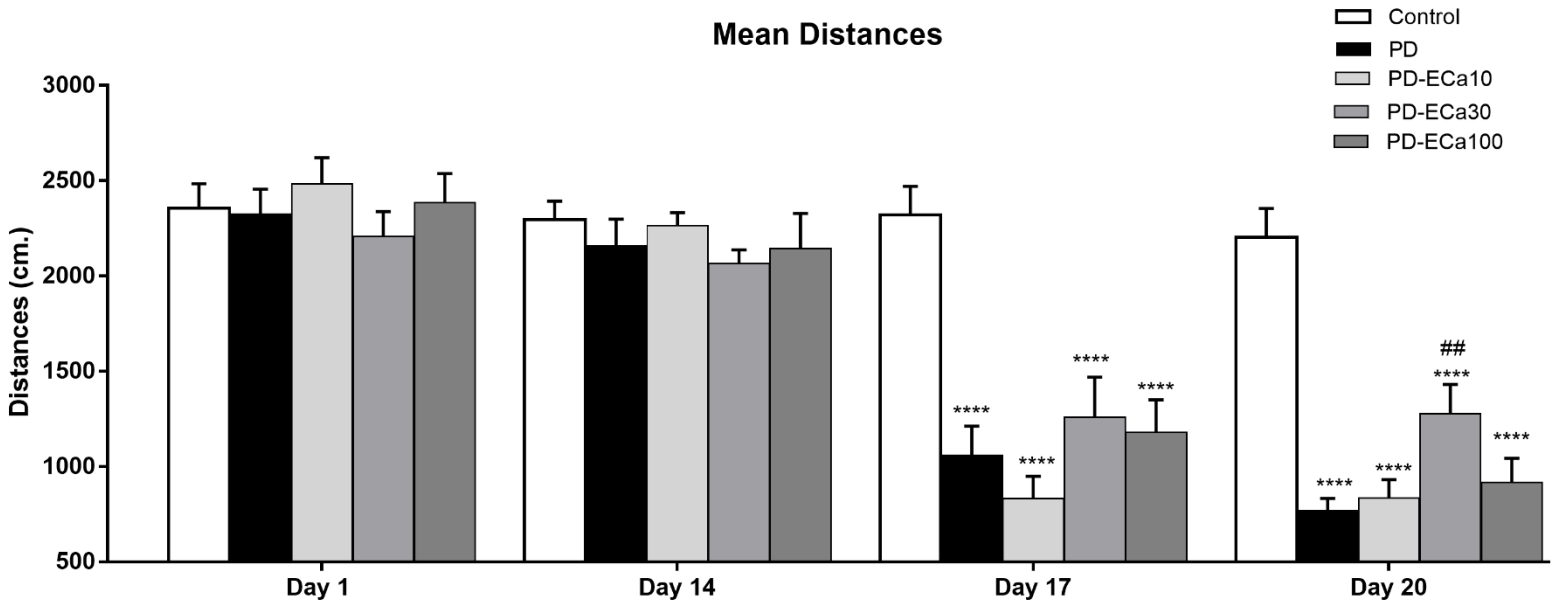


Figure 19 demonstrates the distances of rats on day 14, 17 and 20. *The distances were expressed as mean ± S.E.M. (n=10/group). ****p<0.0001 compared to control group, ##p<0.01 compared to PD (Fisher's LSD post-hoc test).*

4.1.1.2 Locomotion time

The locomotion time of rats was measured on experimental day 1, 14, 17, and 20. At baseline, mean locomotion time of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups were 216.5 ± 10, 228.5± 5.5, 226.3± 4.4, 231± 6 and 233.6± 7.6 s, respectively ($F(4, 45) = 0.88$, $p = 0.4835$, one-way ANOVA). On day 14, the mean locomotion time of rats was not significantly different indicating no motor-stimulating effect of ECa233 in rats ($F(4, 45) = 0.3466$, $p = 0.8450$, one-way ANOVA). The mean locomotion time of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups on day 14 were 207 ± 7.6, 218.3 ± 6.5, 209 ± 6.7, 213 ± 6.2 and 215.4 ± 11 s, respectively. On day 17, the average distances of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 211.8 ± 8.6, 155.7 ± 11.2, 128.6± 12.8, 162.8 ± 15.9 and 160.5 ± 12.3 s, respectively. Rotenone treatment caused motor impairments in rats as locomotion time of rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were significantly lower than that of controls ($p<0.01$, $p<0.0001$, $p<0.01$, and $p<0.01$ respectively, Fisher's LSD post-hoc test). On day 20, the average distances of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 209.4 ± 4.5, 142.7 ± 7, 138.8 ± 12.5, 167.4 ± 12.4 and 140 ± 11.7 s, respectively. One-way ANOVA revealed a significant difference among experimental groups ($F(4, 45) = 8.843$, $p<0.0001$). Rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups showed a significant decrease of mean distances compared to control ($p<0.0001$, $p<0.0001$, $p<0.01$ and $p<0.0001$, respectively, Fisher's LSD post-hoc test) **(Figure 20).**

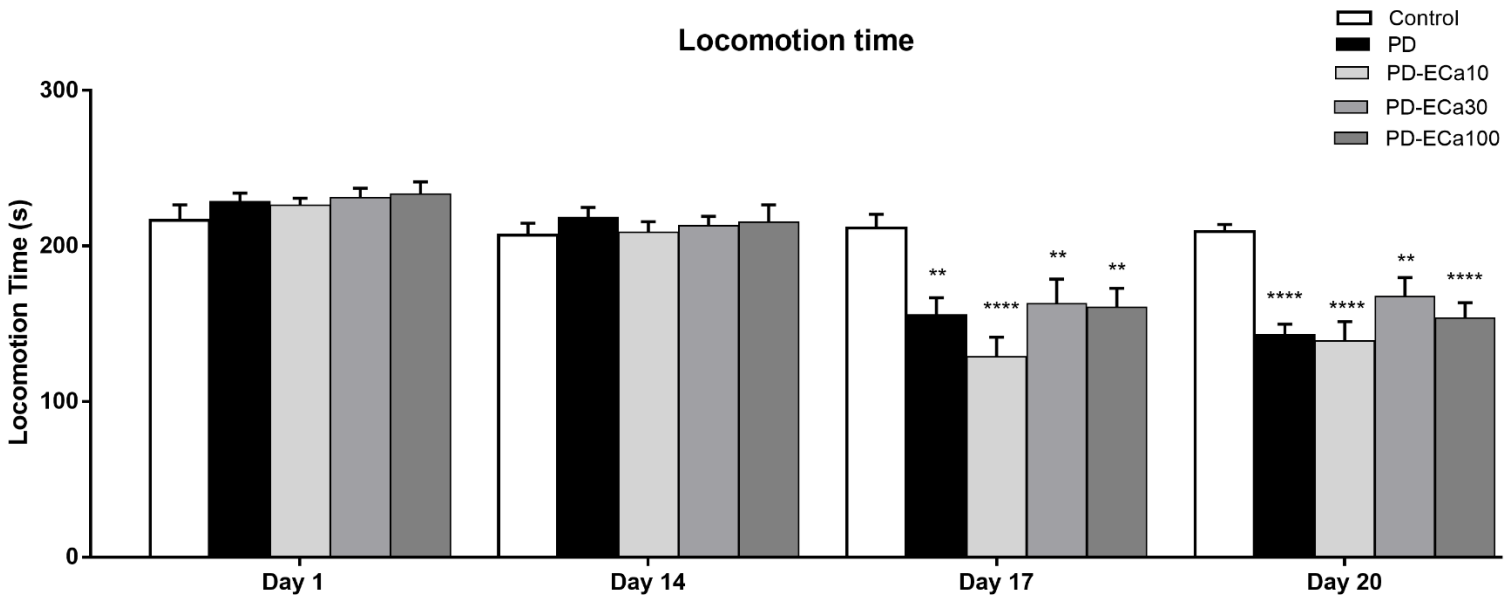


Figure 20 demonstrates the locomotion time of rats on day 14, 17 and 20. The locomotion time were expressed as mean ± S.E.M. (n=10/group). **p<0.01, ****p<0.0001 compared to control, (Fisher's LSD post-hoc test).

4.1.1.3 Resting time

The resting time of rats was measured on experimental day 1, 14, 17, and 20. On day 1, the mean resting time of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 81.5± 8.9, 71.6 ± 5.6, 73.6 ± 4.5, 68.9± 6 and 66.4 ± 7.6 s, respectively. There was no significant difference among the experimental groups at baseline ($F(4, 45) = 0.7428$, $p = 0.5679$). The mean resting time of rats on day 14 showed no motor-stimulating effect of ECa233 as the mean resting time of all groups were not significantly different ($F(4, 45) = 0.2631$, $p = 0.9001$, one-way ANOVA). The average resting time of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups on day 14 were 93 ± 7.6, 83.8 ± 6.5, 91 ± 6.7, 87 ± 6.2 and 84.6 ± 11 s, respectively. On day 17, the mean resting time of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 88.2 ± 8.6, 144.1 ± 11, 171.4 ± 12.8, 136.2 ± 15.3 and 139.5 ± 12.3 s, respectively. One-way ANOVA revealed a significant difference among groups ($F(4, 45) = 6.056$, $p<0.001$). Rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups showed a significant increase of mean resting time compared to control, suggesting motor deficits induced by rotenone ($p<0.01$, $p<0.0001$, $p<0.01$, and $p<0.01$, respectively, Fisher's LSD post-hoc test). Additionally, rats in PD-ECa30 had lower resting time compared to PD-ECa10 ($p<0.05$, Fisher's LSD post-hoc test). On day 20, the average distances of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 90 ± 4, 157.3 ± 7, 161.2 ± 12.5, 132.1 ± 12 and 160 ± 11.7 s, respectively. One-way ANOVA revealed a significant difference among groups ($F(4, 45) = 9.219$, $p<0.0001$). Rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups showed a significant increase of mean resting time compared to control ($p<0.0001$, $p<0.0001$, $p<0.01$ and $p<0.0001$, respectively, Fisher's LSD post-hoc test). Moreover, the mean resting time of PD-ECa30 rats was significantly decreased compared to that of PD-ECa10 rats ($p<0.05$, Fisher's LSD post-hoc test) **(Figure 21)**.

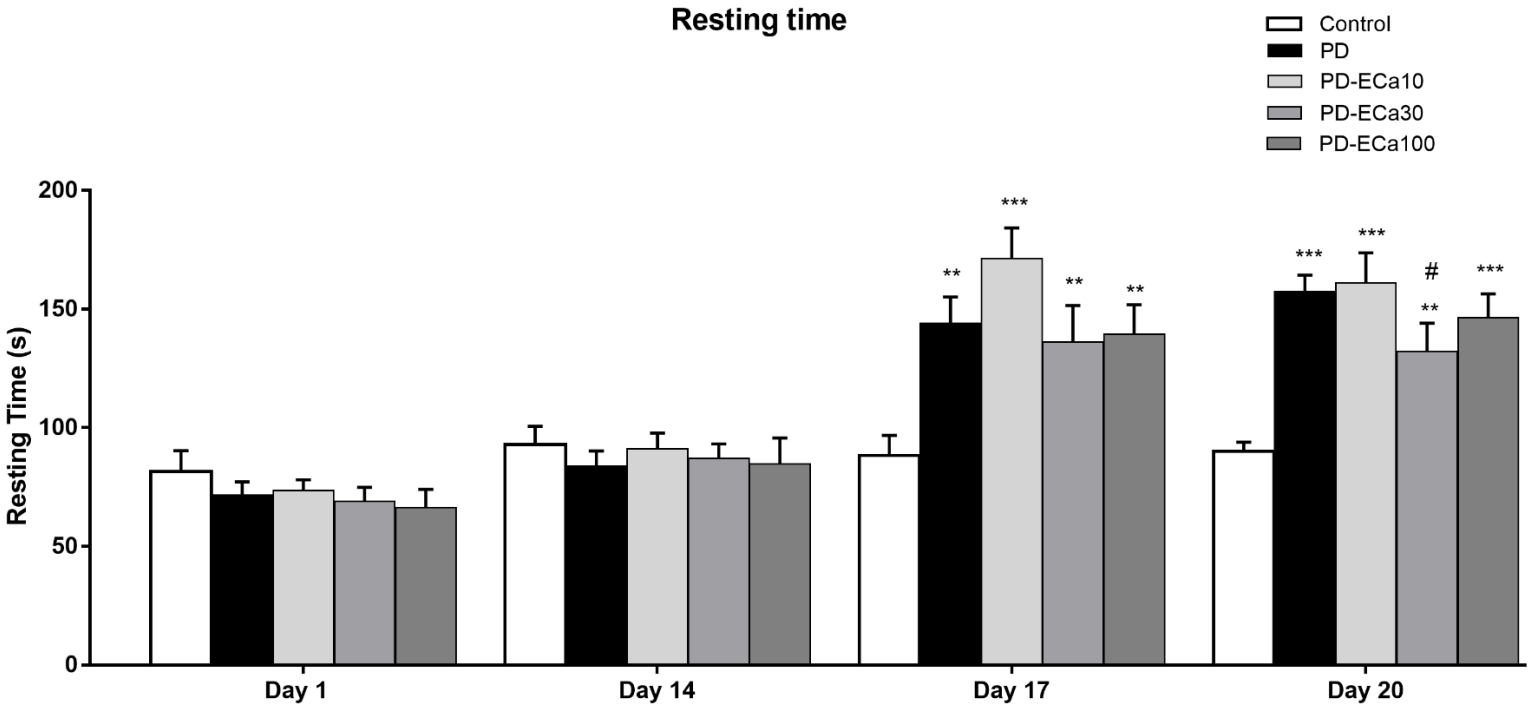


Figure 21 demonstrates the resting time of rats on day 14, 17 and 20. The resting time were expressed as mean ± S.E.M. (n=10/group). **p<0.01, ***p<0.001 compared to control, #p<0.05 compared to PD, Fisher's LSD post-hoc test.

4.1.2 Apomorphine challenge

To determine whether locomotor deficits were a dopamine-dependent, apomorphine, a dopamine agonist, was subcutaneously injected to all rats on day 21. Rat's behaviors in the open field were then measured 5 minutes after injection. The mean distances on the day 21 of rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were significantly higher than those of the same groups on day 20 ($p<0.001$, $p<0.001$, $p<0.05$ and $p<0.001$, respectively, paired t-test) **(Figure 22A)**. There was no significant difference of the mean distances between day 20 and day 21 in the control group.

In the same way, the mean locomotion time of rats in PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups on day 21 were significantly higher than those of the same groups on day 20 ($p<0.01$, $p<0.001$, $p<0.01$ and $p<0.01$, respectively, paired t-test), while there was no change in controls **(Figure 22B)**.

In addition, the mean resting time of rats in PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups on day 21 were significantly lower than those of the same groups on day 20 ($p<0.01$, $p<0.001$, $p<0.01$ and $p<0.01$, respectively, paired t-test) **(Figure 22C)**. There was no significant difference of the mean resting time between day 20 and day 21 in the control group.

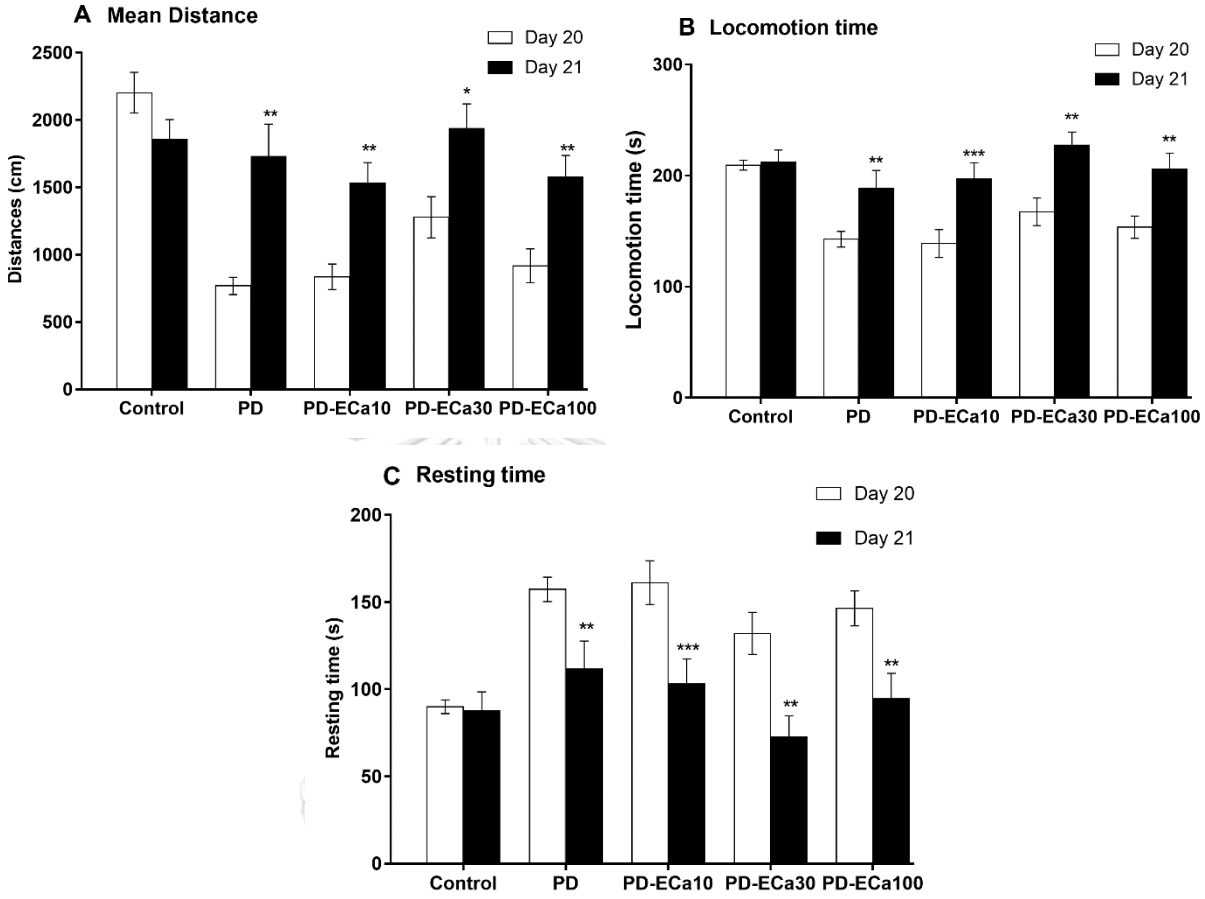


Figure 22 demonstrates the locomotor activities of the rats on the apomorphine challenge day. The distance **(A)**, locomotion time **(B)** and resting time **(C)** were represented as mean ± S.E.M. (n=10/group) *p<0.05, **p<0.01, ***p<0.001; Paired t-test.

4.1.3 TH-positive neurons in the substantia nigra

The dopaminergic neurons in the substantia nigra were stained with anti- tyrosine hydroxylase antibody and observed under the microscope **(Figure 23A)**. The average numbers of TH-positive neurons in the substantia nigra of rats in control, PD, PD-ECa10, PD-ECa30, and PD-ECa100 groups were 720 ± 11.85, 532 ± 28.26, 544.8 ± 42.03, 793.4 ± 50.69 and 650 ± 54.53 cells, respectively **(Figure 23B)**. One-way ANOVA revealed a significant difference among groups ($F(4, 20) = 7.64$, $p=0.0007$). Rats in PD and PD-ECa10 showed significantly lower in the numbers of TH-positive neurons than those of controls ($p<0.01$ and $p<0.01$, respectively, Fisher's LSD post-hoc test) There was no significant difference in the numbers of TH-positive neurons of rats in PD-ECa30 and PD-ECa100 groups compared to those of controls. Furthermore, the numbers of TH-positive neurons in ECa30 rats was significantly higher than that of PD ($p<0.001$, Fisher's LSD post-hoc test), suggesting the protective effect of ECa233 (30 mg/kg) against rotenone-induced dopaminergic neuronal death.

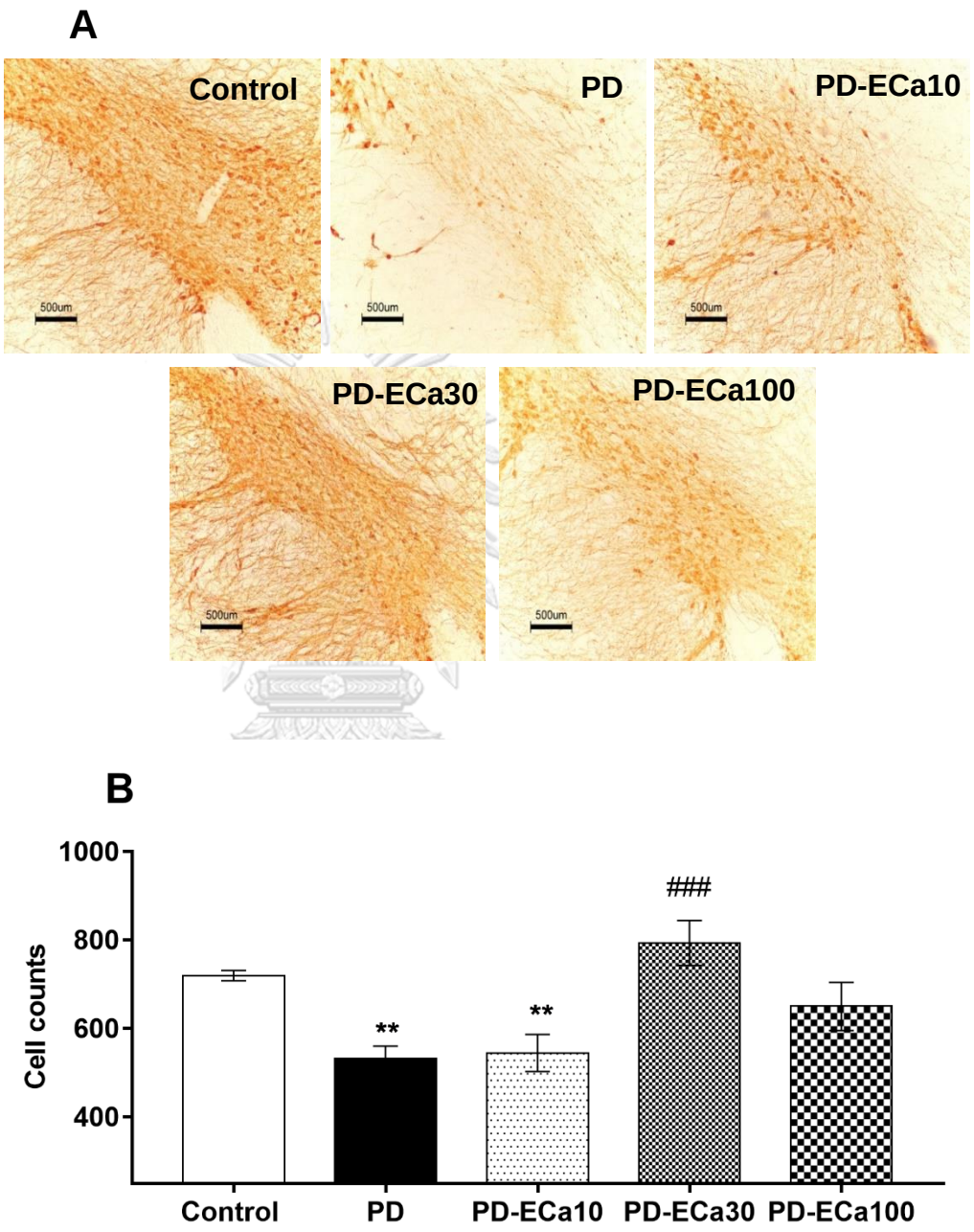


Figure 23 Expression of tyrosine hydroxylase positive neurons in the substantia nigra were observed under a microscope (A). The number of TH-positive neuron in each group was counted individually and expressed as mean ± S.E.M. (n = 5/group) (B). **p<0.01 compared to control, ###p<0.001 compared to PD, Fisher's LSD post-hoc test.

4.1.4 The intensity of TH-positive neurons in the striatum

The dopaminergic fibers in the striatum were stained with anti-tyrosine hydroxylase antibody and observed under the microscope **(Figure 24A).** The mean intensity of TH-positive fibers in the striatum of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups were 23.92 ± 0.9, 17.92 ± 0.48, 18.78 ± 0.64, 23.2 ± 0.68 and 20.11 ± 1.35, respectively **(Figure 24B)**. One-way ANOVA revealed a significant difference among groups ($F(4, 25) = 9.521$, $p<0.0001$). Rats in PD, ECa10, and ECa100 showed significantly lower in the intensity of TH-positive fibers than that of controls ($p<0.0001$ $p<0.001$ and $p<0.01$, respectively, Fisher's LSD post-hoc test). There was no significant difference in the intensity of TH-positive fibers of rats in ECa30 compared to controls. Moreover, the numbers of TH-positive fibers in ECa30 and ECa100 were significantly higher than that of PD ($p<0.0001$, $p<0.05$, respectively, Fisher's LSD post-hoc test), indicating the protective effect of ECa233 against rotenone-induced dopaminergic axonal loss.

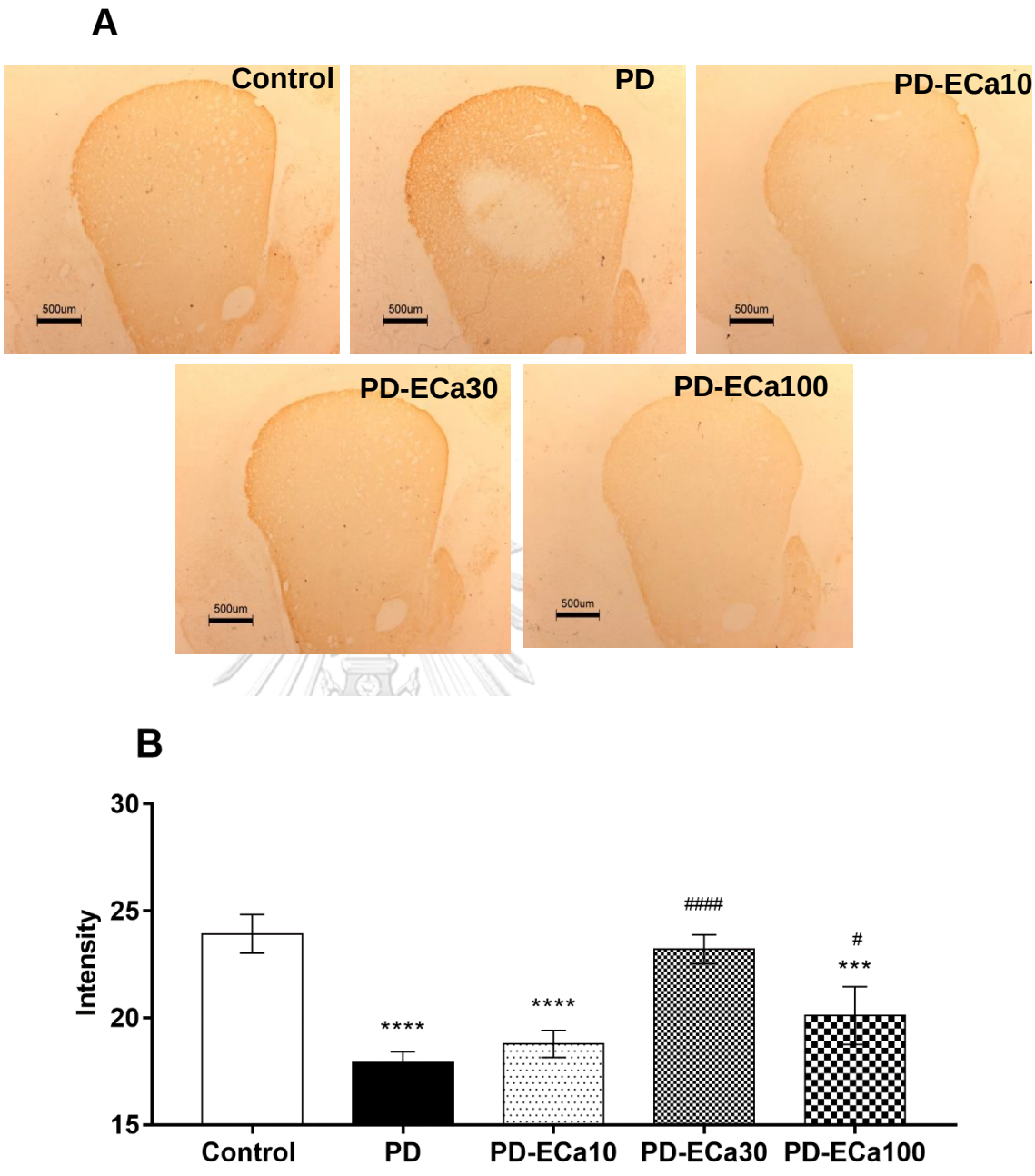


Figure 24 Tyrosine hydroxylase positive fibers in the striatum were observed under the microscope (A). The intensity of TH-positive fibers in each group was measured and expressed as mean ± S.E.M. (n = 5/group) (B). ***p<0.001, ****p<0.0001 compared to control, #p<0.05, ####p<0.0001 compared to PD, Fisher's LSD post-hoc test.

4.1.5 Mitochondrial complex I activity

Rate of mitochondrial complex I enzyme activity of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups were 0.05064 ± 0.011, 0.02141± 0.0038, 0.0226 ± 0.0037, 0.0411 ± 0.0050and 0.01948 ± 0.0014 mOD/s/mg protein, respectively. There was a significant effect of treatments on mitochondrial complex I activity among groups ($F(4, 20) = 5.37$, $p=0.0042$, one-way ANOVA). Mitochondrial complex I activity of rats in PD, PD-ECa10 and PD-ECa100 were significantly lower than that of the controls ($p<0.01$, $p<0.01$ and $p<0.01$, respectively, Fisher's LSD post-hoc test). There was no significant difference of mitochondrial complex I enzyme activity between control and ECa30 rats. Moreover, mitochondrial complex I enzyme activity in ECa30 rats was significantly higher than that of PD rats ($p<0.05$, Fisher's LSD post-hoc test) **(Figure 25)**. The results indicated that ECa233 protected mitochondrial complex I dysfunction induced by rotenone.

4.1.6 MDA levels

Malondialdehyde (MDA) levels in the brain tissues were detected to represent lipid peroxidation levels in the brains. MDA levels of rats in control, PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups were 8.899 ± 0.35, 11 ± 0.56, 10.52 ± 0.53, 9.716± 0.32 and 10.46 ± 0.32 μM/mg protein, respectively. One-way ANOVA revealed a significant difference among groups ($F(4, 20) = 3.68$, $p=0.0211$). MDA levels of rats in PD, ECa10 and ECa100 were significantly higher than that of the controls ($p<0.01$, $p<0.05$ and $p<0.05$, respectively, Fisher's LSD post-hoc test). Brain's MDA levels of PD-ECa30 was not different from control and. Moreover, Brain's MDA level of ECa30 rats was significantly lower than that of PD rats ($p<0.05$, Fisher's LSD post-hoc test) **(Figure 26**).

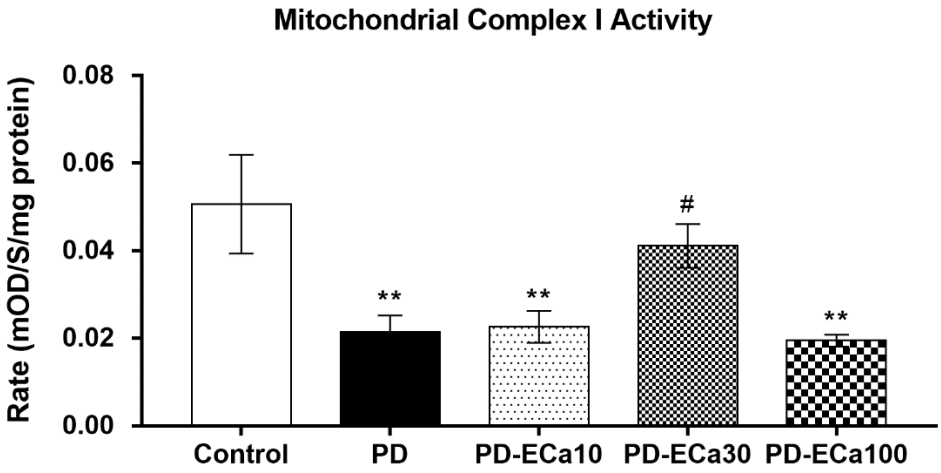


Figure 25 demonstrates the effect of Eca233 on mitochondrial dysfunction induced by rotenone. The data are presented as mean ± S.E.M. (n=5/group). **p<0.01 compared to control group, #p<0.05 compared to PD, Fisher's LSD post-hoc test.

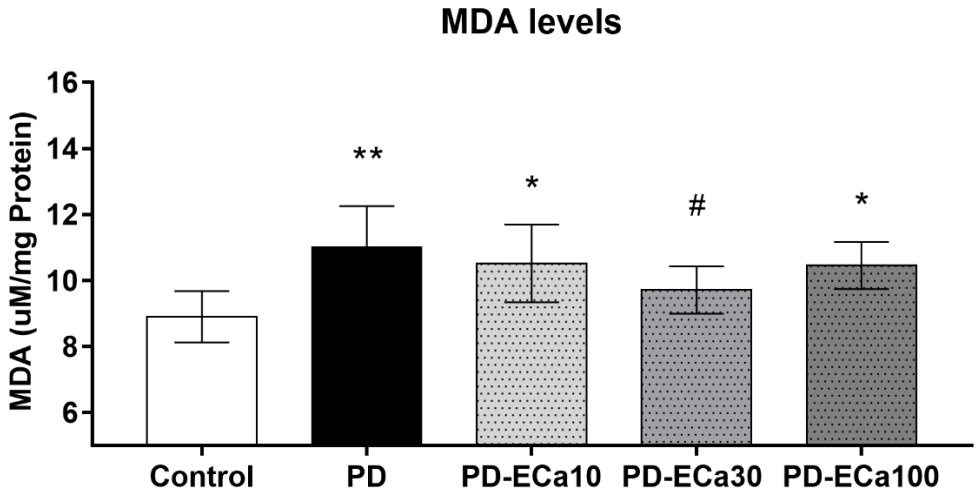
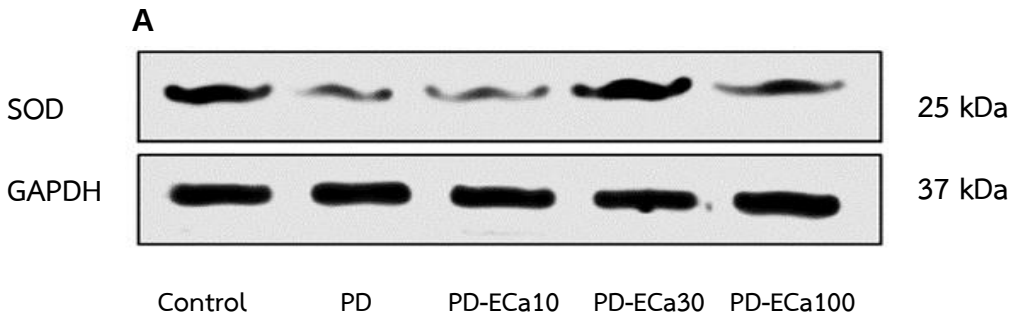


Figure 26 demonstrates the MDA levels in the rat's brains. The data are presented as MDA mean ± S.E.M. (n=5/group). *p<0.05, **p<0.01 compared to control, #p<0.05 compared to PD, Fisher's LSD post-hoc test.

4.1.7 Antioxidant enzymes expression

SOD and catalase expression were measured using western blot technique. There was a significant difference in SOD expression among the groups (F (4, 20) = 3.05, p=0.0409, one-way ANOVA). SOD expression of PD rats was significantly lower than that of controls (p<0.05, Fisher's LSD post-hoc test). In addition, SOD expression of ECa30 rats was significantly higher than that of PD (p<0.01, Fisher's LSD post-hoc test) **(Figure 27)**.

One-way ANOVA revealed a significant difference of catalase expression among groups (F (4, 20) = 2.488, p=0.0762). Catalase expression of PD rats was significantly lower than that of controls (p<0.05, Fisher's LSD post-hoc test). However, catalase expression of rats in PD-ECa30 and PD-ECa100 were significantly higher than that of PD (p<0.05, Fisher's LSD post-hoc test) **(Figure 28**).



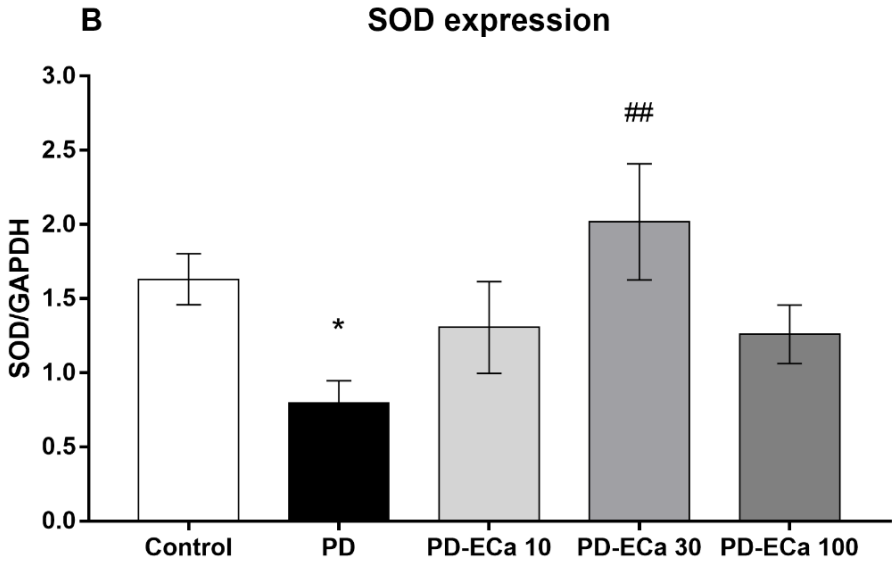
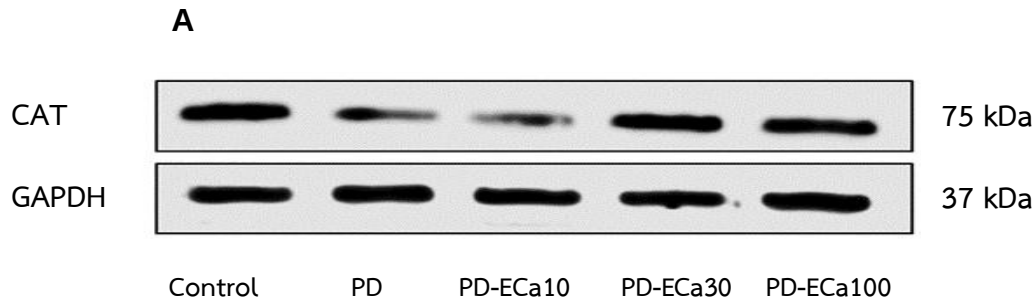


Figure 27 demonstrates the expression of SOD (A) and SOD densities (B) The data are expressed as mean ± S.E.M. (n = 5/group). *p<0.05 compared to control, ##p< 0.01 compared to PD, Fisher's LSD post-hoc test.



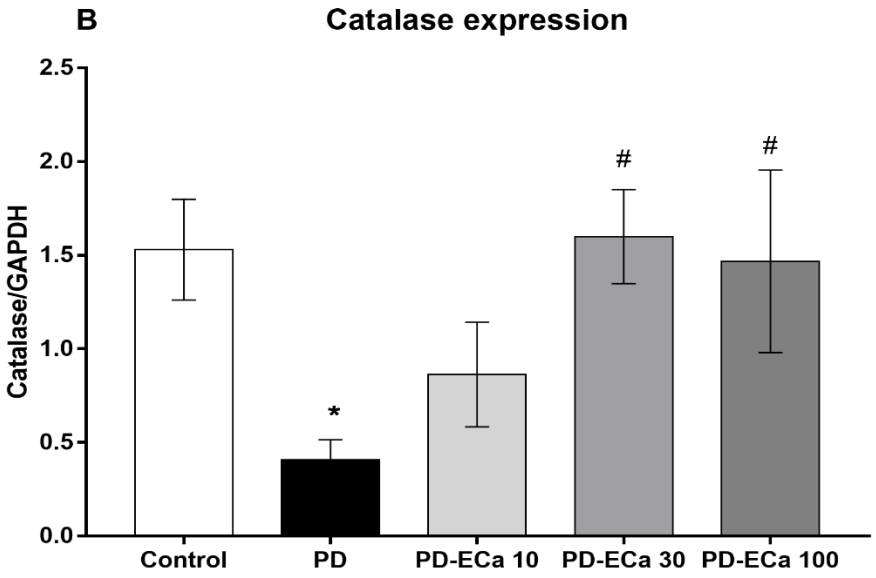


Figure 28 demonstrates the expression of CAT(A) and catalase densities (B). The data are expressed as mean ± S.E.M. (n = 5/group). *p<0.05 compared to control, #p<0.05 compared to PD, Fisher's LSD post-hoc test.

4.2. *In vitro* study

4.2.1 Determination of non-toxic concentrations of ECa233

Cell viability of SH-SY5Y cells was determined after 12-hour incubation with ECa233 5, 10, 25, 50, and 100 µg/mL. There was no significant difference in cell viability among concentrations. The results indicated that ECa233 up to 100 µg/mL has no toxicity to SH-SY5Y cells (**Figure 29)**.

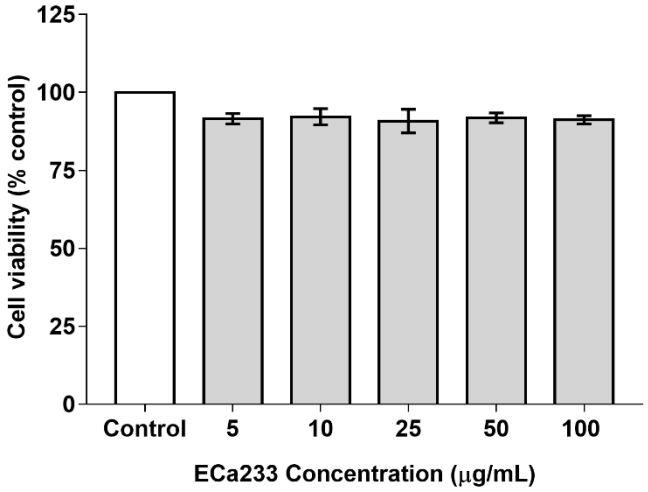


Figure 29 The effect of ECa233 on cell viability. Data are presented as mean ± S.E.M. of three independent experiments.

4.2.2 Determination of toxic concentrations of rotenone

To determine toxic concentrations of rotenone, SH-SY5Y cells were incubated with rotenone for 48 hours. Rotenone (50-1000 µM) significantly reduced cell viability compared to control ($p<0.01$) **(Figure 30)**. Therefore, 500 nM of rotenone was selected for further cytotoxicity induction as it could reduce cell viability to 57.33 ± 3.65%.

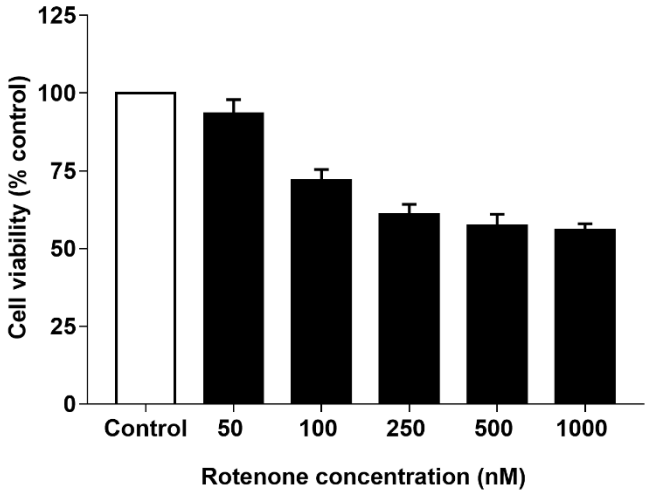


Figure 30 The cytotoxic effect of rotenone on cell viability. Data are presented as mean ± S.E.M. of three independent experiments.

4.2.3 The protective effects of ECa233 against rotenone-induced cytotoxicity

To determine the protective effects of ECa233, cells were pretreated with ECa233 for 12 hours, followed by 500 nM rotenone for 48 hours. Rotenone treatment decreased cell viability significantly compared to controls ($p<0.01$, Tukey's post-hoc test). Pretreatment with ECa233 (10, 25, 50 and 100 µg/mL) significantly increased cell viability compared to rotenone-treated alone ($p<0.01$, Tukey's post-hoc test), indicating the protective effects of ECa233 against rotenone induced-cytotoxicity in SH-SY5Y **(Figure 31)**.

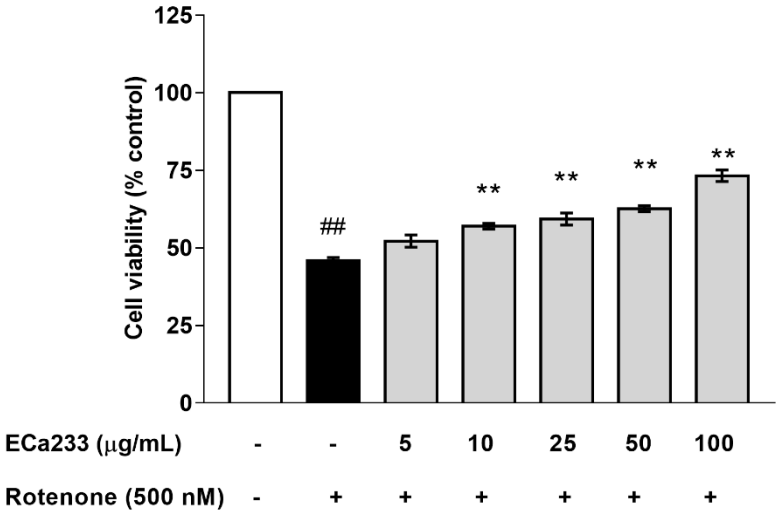


Figure 31 The protective effects of ECa233 against rotenone-induced cytotoxicity in SH-SY5Y cell. Data are represented as mean ± S.E.M. of three independent experiments. ##p<0.01 compared to control, **p<0.01 compared to rotenone treated alone, Tukey's post-hoc test.

4.2.4 The effect of ECa233 on rotenone-induced intracellular ROS levels

Intracellular ROS levels were determined at 6, 24, and 48 hours after rotenone treatment. Rotenone significantly increased intracellular ROS levels at 6 and 24 hours (p<0.05 and p<0.01, respectively), but not 48 hours after treatment. ECa233 (50 and 100 μg/mL) pretreatment significantly decreased intracellular ROS induced by rotenone at 24 hours after rotenone treatment (p<0.05, Tukey's post-hoc test) **(Figure 32)**. This effect of ECa233 was not present at 6 and 48 hours after rotenone incubation.

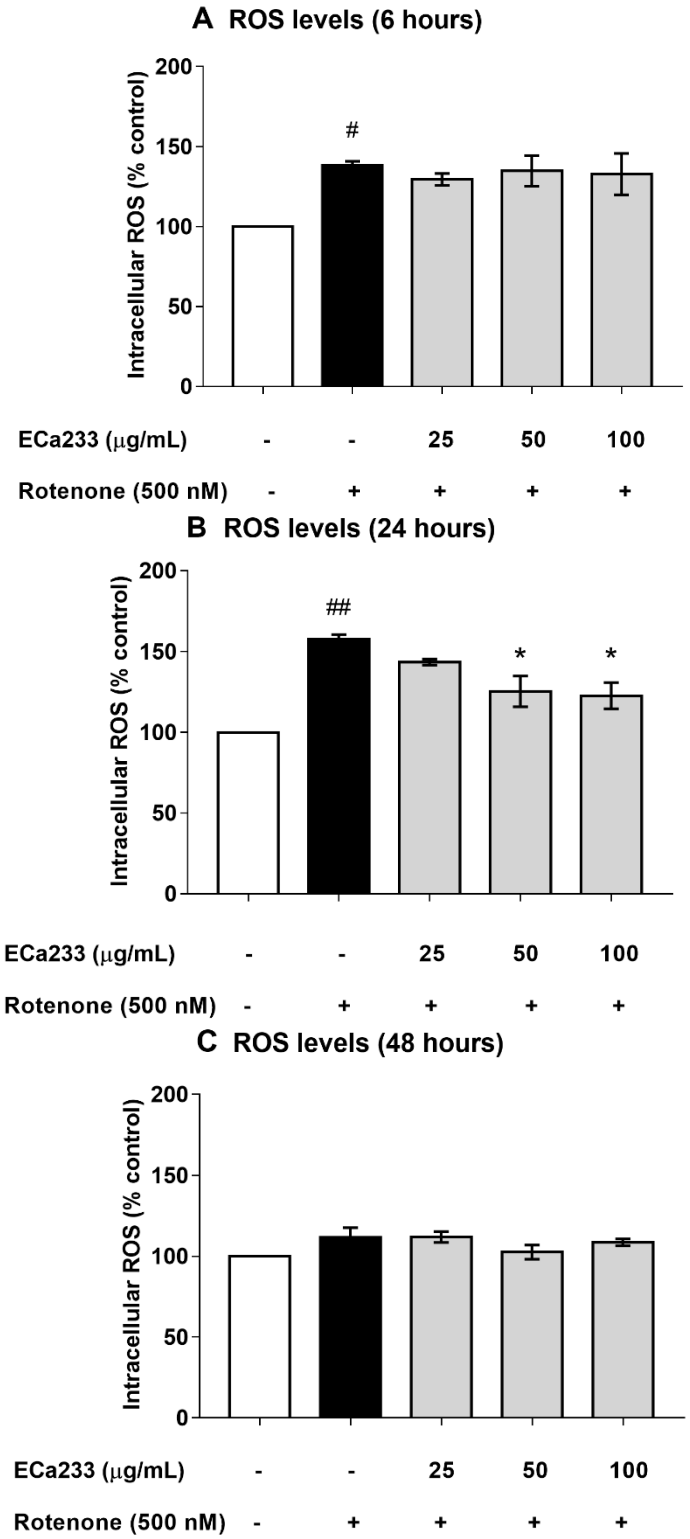


Figure 32 demonstrates intracellular ROS levels at 6 **(A)**, 24 **(B)**, and 48 hours **(C)** after rotenone incubation. Data are represented as mean ± S.E.M. of three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to control, $^{*}p<0.05$ compared to rotenone-treated alone.

4.2.5 The effect of ECa233 on rotenone-induced mitochondrial membrane potential reduction

Mitochondria membrane potentials were measured using TMRE fluorescent intensity. Rotenone significantly decreased TMRE fluorescent intensity compared to control ($p<0.01$). Pretreatment with ECa233 25, 50, and 100 µg/mL significantly increased TMRE fluorescent intensity compared to rotenone-treated alone ($p<0.01$, Tukey's post-hoc test) **(Figure 33).** These results suggested that ECa233 pretreatment prevented against mitochondrial membrane potential loss induced by rotenone.

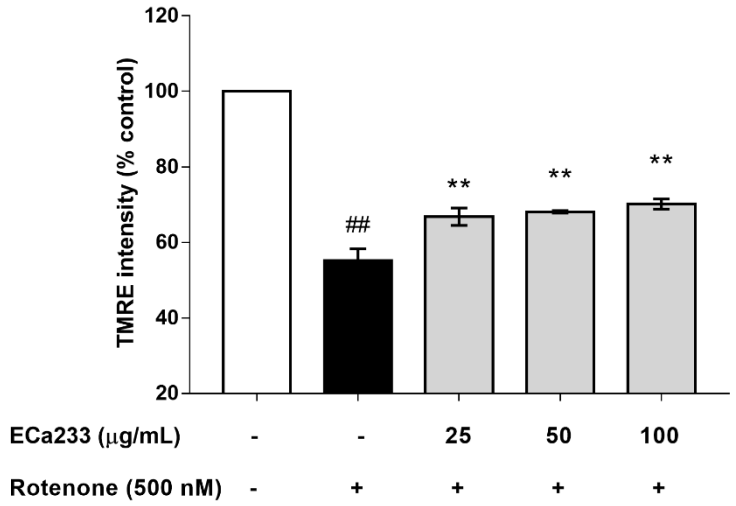


Figure 33 demonstrates the TMRE intensity. Data are presented as mean ± S.E.M. of three independent experiments. ##$p<0.01$ compared to control, **$p<0.01$ compared to rotenone-treated alone.

4.2.6 Mitophagy enhancing effect of ECa233

Mitophagy was detected using Mito dye staining and presented as Mito dye fluorescent intensity. ECa233 (100 µg/mL) treated alone had no effect on mitophagy. Rotenone treatment significantly decreased mitophagy compared to control ($p<0.01$). Pretreatment with ECa233 25, 50 and 100 µg/mL increased mitophagy significantly compared to rotenone-treated alone, ($p<0.05$, $p<0.01$, and $p<0.01$, respectively) **(Figure 34)**.

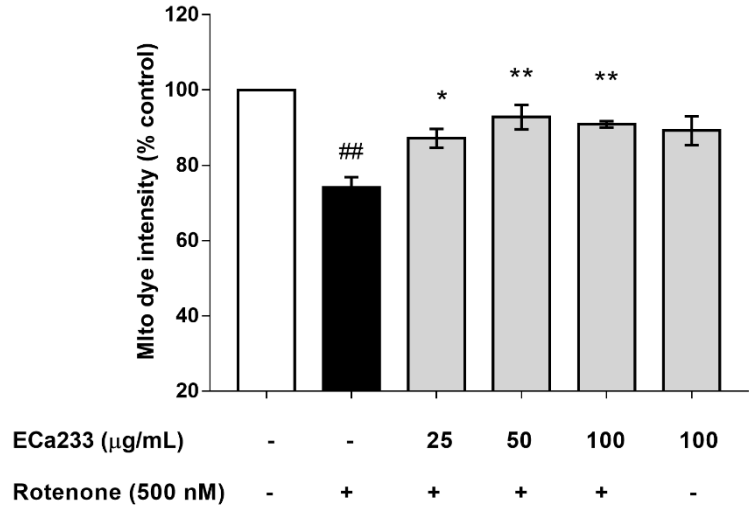
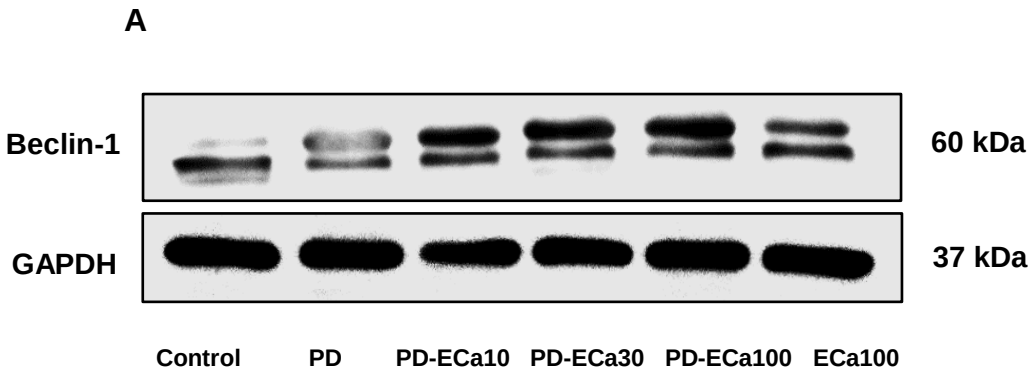


Figure 34 Effects of rotenone and ECa233 on mitophagy. Data are presented as mean ± S.E.M. of three independent experiments. ##p<0.01 compared to control, *p<0.05, ** p<0.01 compared to rotenone-treated alone.

4.2.7 Mitophagy associated protein expression

Beclin-1, LC3-I, and LC3-II protein expressions were measured using western blot technique. ECa233 (100 μg/mL) did not affect Beclin-1 protein expression. Rotenone treatment tended to decrease Beclin-1 protein expression, but this effect was not significantly different from control. ECa233 (50 and 100 μg/mL) pretreatment significantly increased Beclin-1 protein expression (p<0.05 and p<0.01, respectively) compared to retenone-treated alone **(Figure 35)**.

ECa233 (100 μg/mL) had no effect on mitophagy LC3II/LC3I ratio. Rotenone treatment tended to increase LC3II/LC3I ratio. ECa233 (50 and 100 μg/mL) significantly increased LC3II/LC3I ratio compared to rotenone-treated alone (p<0.05) **(Figure 36)**.



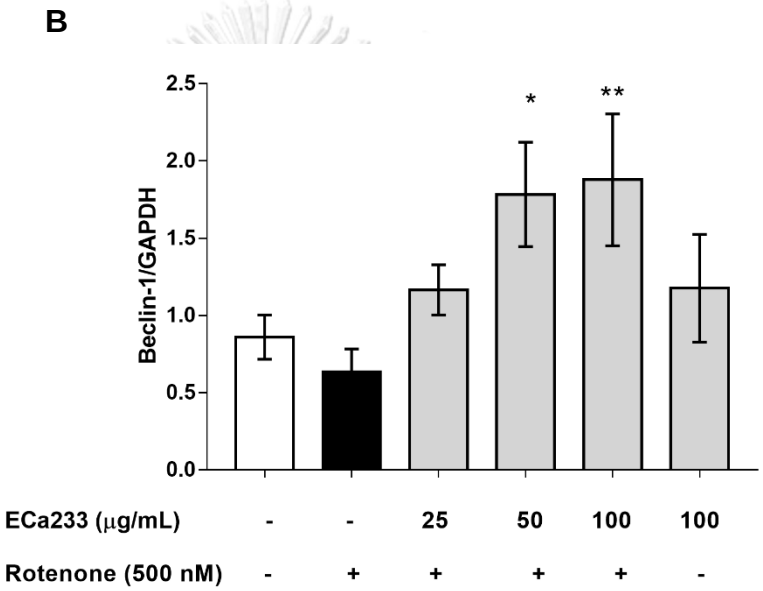
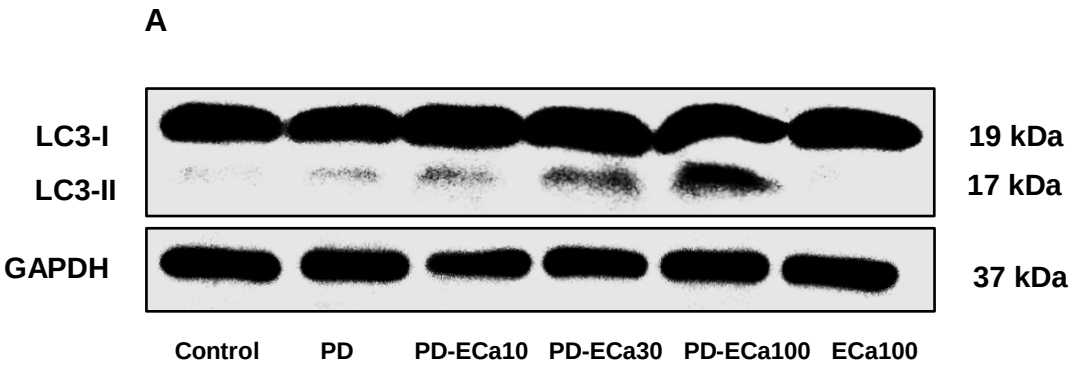


Figure 35 demonstrates the expression of Beclin-1 **(A)** and Beclin-1 densities **(B)**. Data are expressed as mean ± S.E.M. of three independent experiments. *p<0.05, **p<0.01 compared to rotenone-treated alone, Fisher's LSD post-hoc test.



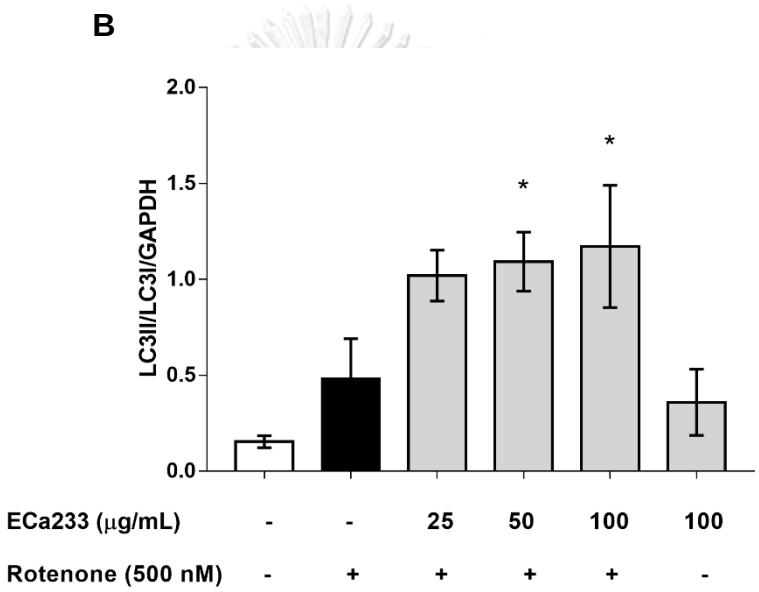


Figure 36 demonstrates the expression of LC3-II and LC3-I **(A)** and LC3-II/LC3-I ratio **(B)**. Data are expressed as mean ± S.E.M. of three independent experiments. *p<0.05 compared to rotenone-treated alone, Fisher's LSD post-hoc test.

4.2.8 Apoptotic protein expression

The expressions of Bcl-2, Bax, and caspase-3 were measured using western blot technique. While rotenone treatment did not significantly decreased Bcl-2 expression, the expression of Bcl-2 in ECa233 (50 and 100 μg/mL) pretreated cells were significantly higher than that of rotenone-treated alone ($p<0.01$) **(Figure 37)**.

Rotenone treatment significantly increased Bax expression ($p<0.01$), while ECa233 (25, 50 and 100 μg/mL) significantly reduced Bax expression compared to rotenone-treated alone ($p<0.05$, $p<0.05$ and $p<0.01$, respectively) **(Figure 38)**.

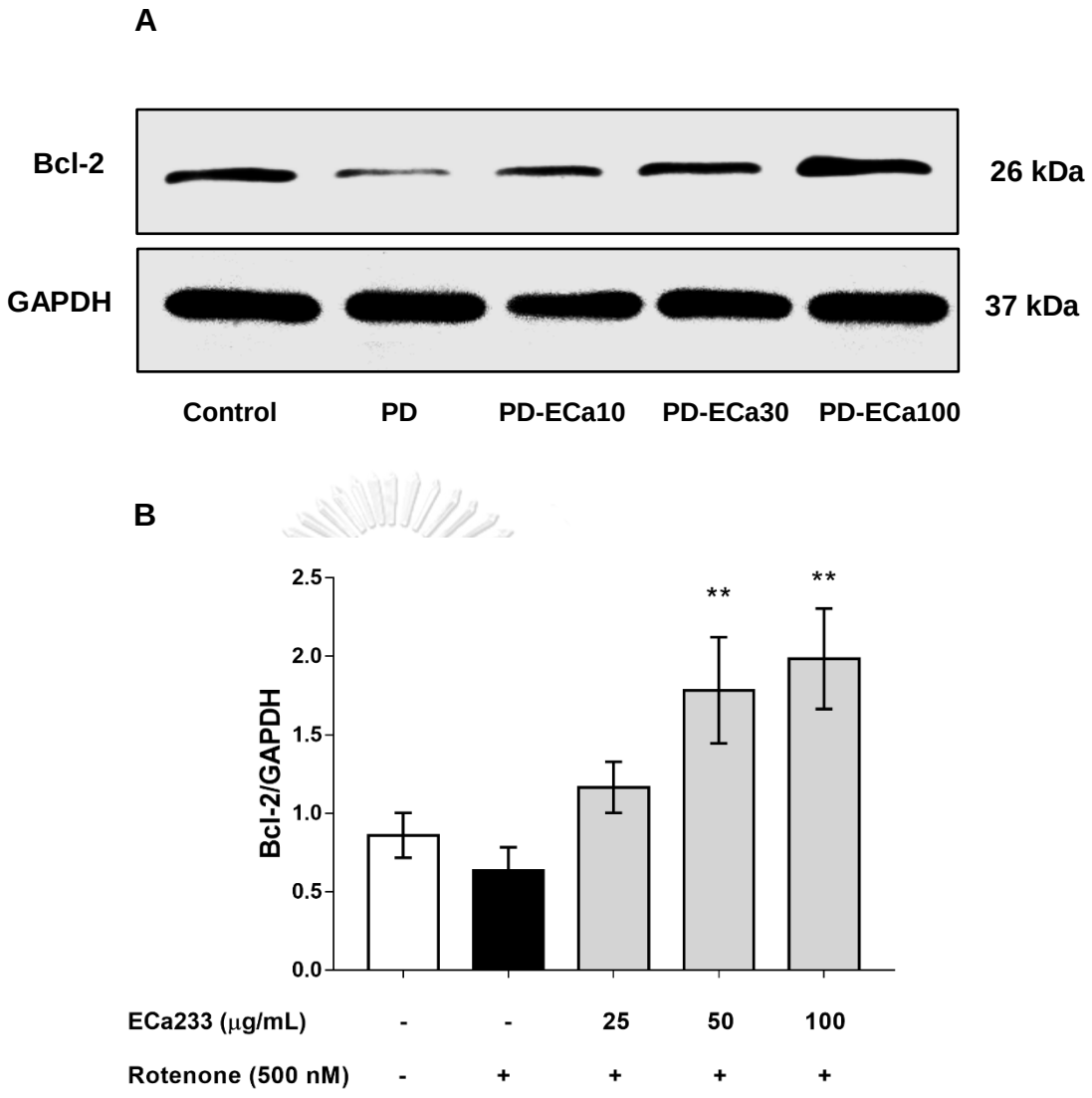


Figure 37 demonstrates the expression Bcl-2 ***(A)*** *and LC3-II/LC3-I ratio* ***(B)***. demonstrates the /GAPDH densities. Data are expressed as mean ± S.E.M. of three independent experiments. **p<0.01 compared to rotenone-treated alone, Fisher's LSD post-hoc test.

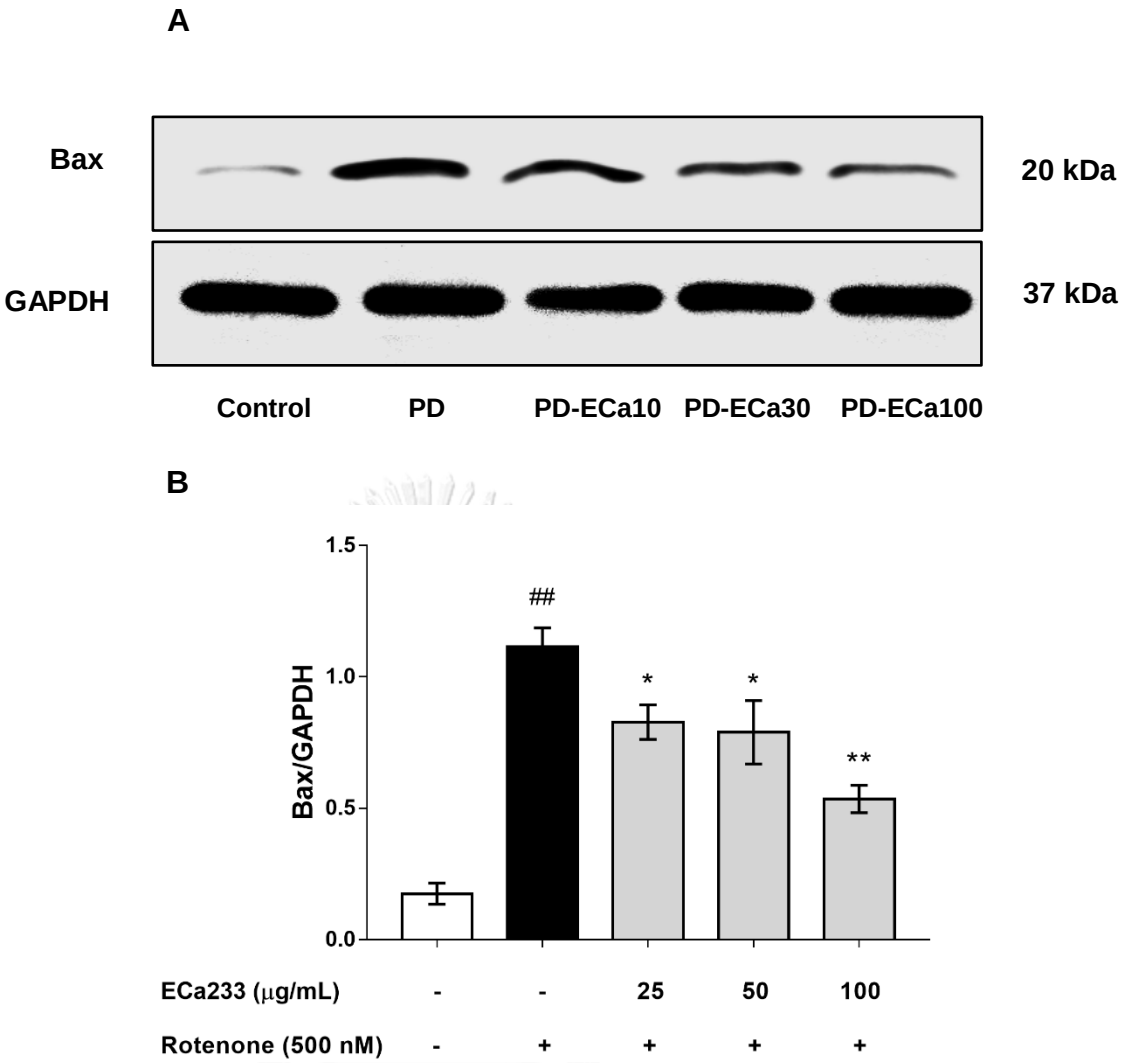


Figure 38 demonstrates the Bax/GAPDH densities. Data are expressed as mean ± S.E.M. of three independent experiments. $^{\#\#}p<0.01$ compared to control, $^{*}p<0.05$, $^{**}p<0.01$ compared to rotenone-treated alone, Fisher's LSD post-hoc test.

The markers of apoptosis, caspase-3, and cleaved-caspase 3 were measured using western blot technique. Rotenone treatment significantly increased cleaved-caspase3/caspase-3 ratio while ECa233 (25, 50 and 100 ug/mL) have a significantly lower of cleaved-caspase3/casepase-3 expression than that of rotenone-treated alone ($p<0.01$) **(Figure 39)**.

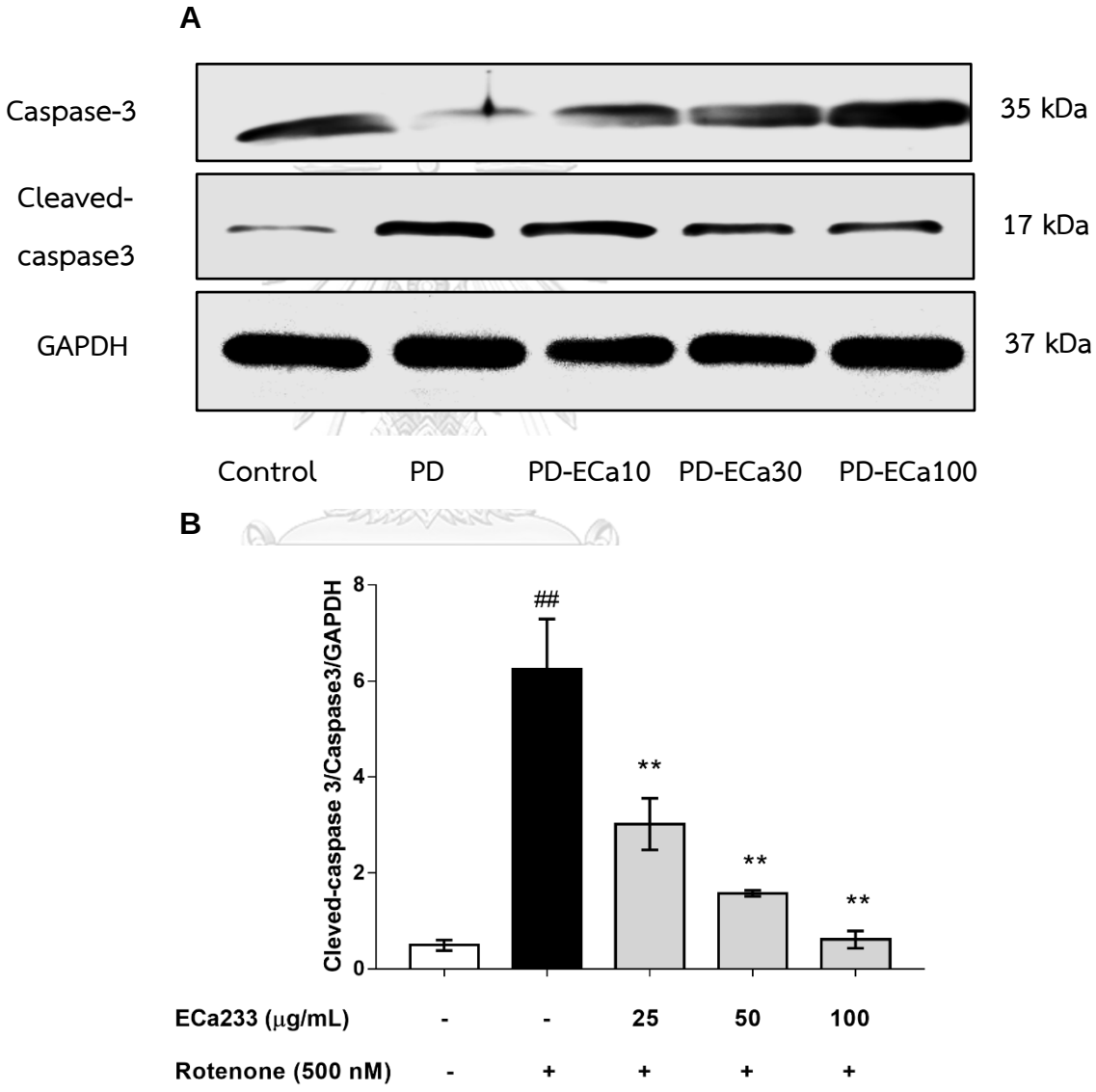


Figure 39 demonstrates the cleaved-caspase 3/caspase 3 densities. Data are expressed as mean ± S.E.M. of three independent experiments. $^{\#\#}p<0.01$ compared to control, $^{**}p<0.01$ compared to rotenone-treated alone.

CHAPTER 5
DISCUSSION AND CONCLUSION

The present study aimed to investigate the neuroprotective effects and the underlying mechanisms of the standardized extract of *Centella asiatica* ECa233 against rotenone model of Parkinson's disease *in vivo* and *in vitro*. It was showed that ECa233 pretreatment ameliorated locomotor deficits and dopaminergic neuronal loss in rats. The neuroprotective effects of ECa233 mediated through mitochondrial complex I protection, the decrease of lipid peroxidation and the increases of SOD and catalase expression in rat brains. In the *in vitro* study, ECa233 preserved cell viability in SH-SY5Y cells. The neuroprotective effects were intracellular ROS reduction, mitochondrial membrane potential protection and mitophagy enhancement.

5.1 ECa233 exhibits neuroprotective effects in rotenone-induced rat model of Parkinson's disease

Rotenone was used in this study to generate parkinsonism behaviors in rats. Rats pretreated with ECa233 (30 mg/kg) showed a significant improvement in locomotor activities compared to rotenone-treated alone, indicating that ECa233 protected against rotenone-induced locomotor impairment. These findings are in consistent with previous studies reporting that asiaticoside and madecassoside improved motor performance in MPTP-induced parkinsonism rats (Xu et al., 2013; Xu et al., 2012). Pharmacokinetic studies revealed the presence of madecassoside and asiaticoside in

brain tissues after single and multiple dosing of ECa233. (Anukunwithaya et al., 2017b, Hengjumrut et al., 2017). Therefore, madecassoside and asiaticoside could be accounted for the protective effect of ECa233 in rotenone-induced parkinsonism in this study.

Loss of dopaminergic neurons in the nigrostriatal pathway is the main pathology in Parkinson's disease (Connolly et al. 2014). The decrease in TH-positive neurons in the substantia nigra and the axon intensities in the striatum of rats receiving rotenone were observed in the present study, while pretreatment of ECa233 (30 mg/kg) preserved both lesions. Therefore, all the behavioral parameters improved in rats receiving ECa233 (30 mg/kg) is due to an increase of viability of neurons in the nigrostriatal pathway. It was notable that a higher dose of ECa233 (100 mg/kg) had a subtle effect in the protection of dopaminergic neurons in the substantia nigra but significantly preserved dopaminergic axon terminals in the striatum. This might be due to the neuritogenic effect of ECa233 seen previously in IMR-32 neuroblastoma cells (Wanakhachornkrai et al., 2013). A recent study also showed that madecassoside, asiaticoside, madecassic acid and asiatic acid increased percent neurite bearing cell and neurite length in N2a cells (Nalinratana et al., 2018). The increase in axon density results in the higher dopamine release at the synapse and subsequently reversed parkinsonism behaviors. As the survival of cell bodies and axonal fibers can be mediated through separate mechanisms (Ries et al., 2008). Therefore, the ability to promote axonal regeneration might be another neuroprotective mechanism of ECa233.

The motor symptoms in Parkinson's disease link to the loss of dopaminergic neurons projecting from the substantia nigra to the striatum. In this study, ECa233 (30 mg/kg) preserved dopaminergic neurons and axonal fibers in the nigrostriatal pathway while the locomotor activity of PD-ECa rats seem not to be fully recovered compared to control rats. This might be due to the systemic toxicity of rotenone causing health-related locomotor problems. Previous study has revealed that chronic rotenone administration in rats caused severe weight loss , hypokinetic behaviors, enlarged stomach, liver necrosis and severe muscle mass loss (LAPOINTE et al., 2004). Therefore, systemic toxicity of rotenone could be accounted for the uncorrelated results seen in behavior and histological study.

Mitochondrial dysfunction plays an important role in the progress of Parkinson's disease (Winklhofer & Haass, 2010). Deficiency in mitochondrial NADH dehydrogenase (Complex I) activity has been reported in the substantia nigra as well as platelets and lymphocytes of Parkinson's disease patients (Schapira et al., 1990). Therefore, protecting mitochondria function is the promising strategy for the treatment of Parkinson's disease. Previous studies showed that madecassoside and asiatic acid could protect against mitochondrial membrane potential reduction induced by H_2O_2 and rotenone *in vitro*, respectively (Bian et al., 2012, Nataraj et al., 2017). Our study is the first to demonstrate the protection of mitochondrial complex I enzyme activity against rotenone toxicity *in vivo*.

An increase in oxidative stress and a decrease in protective mechanisms contribute to cell death in Parkinson's disease (Ogunro et al., 2014). ECa233 decreased lipid peroxidation and increased antioxidant enzyme expression in rotenone models. This

finding is in agreement with previous studies, which reported the antioxidative effects of *C. asiatica* extract, madecassoside and asiaticoside in MPTP models (Bhatnagar et al., 2017, Haleagrahara and Ponnusamy, 2010, Xu et al., 2012, Xu et al., 2013). The protection of mitochondrial function possibly contributes to the reduction of oxidative stress.

The neuroprotective effects of ECa233 appeared to be dose independent. ECa233 (30 mg/kg) markedly alleviated rotenone-induced toxicity, while the subtle effect of ECa233 (100 mg/kg) was presented. The roles of efflux transporters may explain this observation as madecassoside is a substrate of p-glycoprotein and multi-drug-resistant protein (Leng et al., 2013). In addition, asiatic acid, a component of *C. asiatica* extract and a metabolite of asiaticoside, could induce p-glycoprotein expression *in vitro* (Abuznait et al., 2011). Furthermore, Anukunwithaya et al. (2017a) have reported the increase of *Abcb1a* and *Abcc2* mRNA expressions in the small intestine of rats receiving ECa233 100 mg/kg for seven consecutive days. Taken together, the induction of multi-drug-resistant genes and the role of efflux transporter can lead to a subtle effect of ECa233 100 mg/kg.

5.2 ECa233 protected against neurotoxicity of rotenone in SH-SY5Y

In vivo results demonstrated the neuroprotective effects of ECa233 against rotenone toxicity in rats. Madecassoside and asiaticoside could be accounted for the protective effects of ECa233 as they were found in brain of rats after single and multiple dosing of ECa233 (Anukunwithaya et al., 2017b; Hengjumrut et al., 2017). The results in animals demonstrated that neuroprotective

mechanisms of ECa233 are mediated through mitochondria protection and antioxidative effect. Therefore, to gain insight the neuroprotective mechanisms of ECa233, we further investigated molecular neuroprotective mechanisms of ECa233 *in vitro* using SH-SY5Y cells.

Overproduction of ROS and oxidative stress are associated with the pathogenesis of Parkinson's disease. Previous study demonstrated that scavenging of ROS could slow the progress of the neurodegenerative diseases, including Parkinson's disease (Bayani et al., 2009). Therefore, regulating ROS generation and oxidative stress are an effective therapeutic strategy in Parkinson's disease. Our study demonstrated the reduction of intracellular ROS levels induced by rotenone at 24 hours but not 6 and 48 hours. Therefore, we hypothesize that the protective mechanism of ECa233 is not mediated through the direct scavenging mechanism as a direct antioxidant scavenger is supposed to decrease ROS levels at an early hour (Dinkova-Kostova & Talalay, 2008). One possible neuroprotective mechanism is the induction of antioxidant enzymes as seen previously in the *in vivo* study. Moreover, previous studies also demonstrated that *Centella asiatica* extract, asiaticoside, asiatic acid and madecassoside increased the expression of Nrf2, a transcription factor involving the upregulation of antioxidant gene (Gray et al., 2017; Jiang et al., 2017; Wang et al., 2018). Therefore, the activation of Nrf2 could be the key signal of ECa233-induced cytoprotective against rotenone tocixity.

The accumulations of toxic proteins and defected organelles especially defected mitochondria have been proposed as the important pathology of Parkinson's disease (Ryan et al., 2015). Defected mitochondria are the main source of intracellular ROS generation (Winklhofer & Haass, 2010). Basal expression of mitophagy acts as one of the cytoprotective mechanisms and participates in

maintaining cellular homeostasis (Hara et al., 2006; Dadakhujaev et al., 2010). Previous studies reported the decreases of mitophagy levels and its protein markers, Beclin-1 and LC3II/I, in SHSY5Y and PC12 cells after rotenone treatment (Wu et al., 2015; Xiong et al., 2013; Yuan et al., 2015), which are in accordance with our study. Pretreatment with ECa233 enhanced mitophagy against rotenone toxicity and increased the expression of autophagy-associated protein Beclin-1 and LC3II/LC3I ratio. As dysfunctional mitochondria are the main sources of ROS in cells, removing defected mitochondria via induction of mitophagy is proposed as one of neuroprotective mechanism of ECa233.

Pretreatment with ECa233 protected against rotenone-induced mitochondrial membrane potential reduction. The decrease of mitochondrial membrane potential can initiate mitochondria-dependent apoptosis and mitophagy (Anding & Baehrecke, 2017; Martinez-Vicente, 2017). Therefore, modulating mitochondrial membrane potential is benefit for protecting neuronal death. Previously, *Centella asiatica* extract treatment stabilized voltage-dependent anion channel (VDAC) in the open state, causing neuroprotection in N2a cells (Tewari et al., 2016). As the loss of mitochondrial membrane potential is the result of the opening of VDAC, we hypothesize that another protective mechanism of ECa233 might involve the modulation of mitochondrial membrane potential via VDAC. However, to clarify the mechanism of how ECa233 modulate VDAC-related mitochondrial membrane potential reduction, further study should be conducted.

Neuronal apoptosis is one of the important pathological features of Parkinson's disease. To further explore the mechanism of neuroprotection of ECa233, apoptosis-associated proteins including

Bcl-2, Bax, and caspase-3 were measured. In the present study, Bcl-2 expression was increased following ECa233 treatment while Bax expression was significantly decreased. This increased Bcl-2 levels can counteract Bax by forming a heterodimer preventing the mitochondrial-dependent apoptosis (Kobayashi et al., 1998). The result was in accordance with previous studies showing that asiatic acid and asiaticoside prevented neuronal apoptosis by the increase of Bcl-2 and the decrease of Bax proteins (Nataraj et al., 2017; Sun et al., 2015). In this study, ECa233 pretreatment caused the reduction of cleaved-caspase3/caspase3 ratio resulting in the anti-apoptotic effects of ECa233. Therefore, our study has demonstrated that ECa233 can alleviate rotenone-induced neuronal cell death by regulating apoptosis regulator BCL-2 family proteins.

5.3 Conclusion

We found that pretreatment of ECa233 not only improved the movement dysfunction induced by rotenone but also preserved the dopaminergic neurons and their axonal fibers in the nigrostriatal pathway. The neuroprotective mechanisms of ECa233 *in vivo* are involved in the protection of mitochondrial complex I activity, a decrease in lipid peroxidation and the elevation of antioxidant enzyme expression. For the molecular mechanisms of ECa233, pretreatment of ECa233 preserved cell viability via antioxidative effect, mitophagy enhancing effect, the increase of anti-apoptotic protein and the decrease of pro-apoptotic proteins. Therefore, ECa233 should be developed as a therapeutic agent against Parkinson's disease. To gain insight into other molecular mechanisms of ECa233, we suggest investigating survival signaling pathway in Parkinson's disease-precision models in the future.

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APPENDIX

APPENDIX 1

Animal Use Certificate

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ **Original** ☐ **Renew**
Animal Use Protocol No. 15-33-004	**Approval No. 15-33-004**

Protocol Title
Neuro- and hepatoprotective effects of standardized extract of *Centella asiatica* ECa233 in rotenone-induced parkinsonism rat model

Principal Investigator

Ratchanee RODSIRI, PhD

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
May 12, 2015	May 12, 2018

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title PORNCHAI ROJSITTHISAK, **Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

Renamed as amendment requested and approved on December 22nd, 2015

APPENDIX 2

Publication

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Original Article

Neuroprotective effect of a standardized extract of *Centella asiatica* ECa233 in rotenone-induced parkinsonism rats

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ABSTRACT

Background: Mitochondrial dysfunction and reactive oxygen species (ROS) generation cause dopaminergic neurodegeneration in Parkinson's disease. The neuroprotective approach is a promising strategy to slow disease progression in Parkinson's disease. A standardized extract of *Centella asiatica* ECa233 has been previously reported to have pharmacological effects in the central nervous system.

Purpose: This study aimed to determine the neuroprotective effect and mechanisms of ECa233 in rotenone-induced parkinsonism rats.

Methods: Rats were orally given either vehicle or ECa233 (10, 30 and 100 mg/kg) for 20 consecutive days. Rotenone (2.5 mg/kg i.p.) was given to parkinsonism (PD) and ECa-treated rats from day 15 to 20. Locomotor activity was recorded on day 1, 14, 17 and 20. Tyrosine-hydroxylase (TH) immunohistological staining was used to determine dopaminergic neurons in the substantia nigra and striatum. Furthermore, mitochondrial complex I activity, malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase protein expression were measured in brain tissue.

Results: Rats receiving ECa233 30 mg/kg showed a significant increase in distances ($p < 0.01$) together with a higher number and intensity of dopaminergic neurons in the substantia nigra and striatum ($p < 0.001$) compared to PD rats. ECa233 (30 mg/kg) protected against mitochondrial complex I inhibition, decreased MDA levels ($p < 0.05$) and increased SOD ($p < 0.01$) and catalase ($p < 0.05$) expression.

Conclusion: ECa233 can protect against rotenone-induced motor deficits and dopaminergic neuronal death. These effects are mediated through the protection of mitochondrial complex I activity, the effects of antioxidants and the enhancement of antioxidant enzyme expression.

Introduction

Parkinson's disease is a neurodegenerative disease caused by loss of dopaminergic neurons in the substantia nigra resulting in movement disorders (Johnson and Bobrovskaya, 2015). The current pharmacotherapy of Parkinson's disease aims to increase brain dopamine levels to improve motor functions. However, neuroprotective approaches are still under investigation. Various natural compounds have been reported to have neuroprotective effects in Parkinson's disease models (Zhang et al., 2017).

Mitochondrial dysfunction is hypothesized to be one of the molecular mechanisms of neurodegeneration in Parkinson's disease (Shadrina et al., 2010). Various mitochondrial complex I inhibitors including rotenone have been used to produce animal models of Parkinson's disease (Duty and Jenner, 2011). The inhibition of mitochondrial electron transport chain causes ATP insufficiency and reactive oxygen species (ROS) generation, leading to dopaminergic neural death and Parkinsonism behaviors (Martizez and Greenamyre, 2012).

Abbreviations: ATP, adenosine-5'- triphosphate; CMC, carboxymethylcellulose; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; MDA, malondialdehyde; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NP-40, nonylphenoxypolyethyleneoxy ethanol; PBS, phosphate buffered saline; PD, parkinsonism; PFA, paraformaldehyde; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species; TEP, 1, 1, 3, 3-tetraethoxypropane; TH, tyrosine-hydroxylase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride

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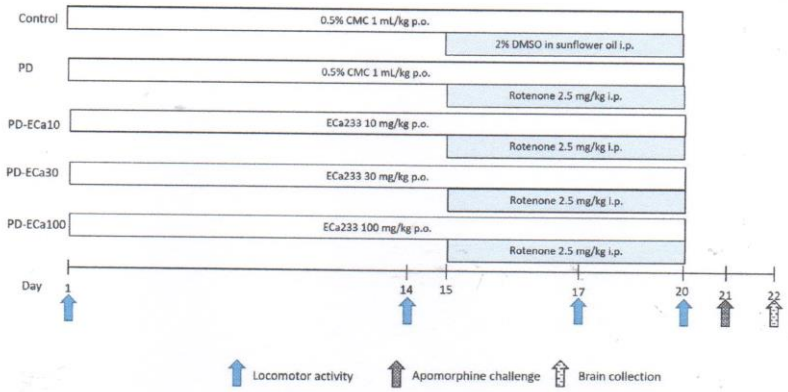

Fig. 1. Treatment groups and experimental timeline.

Therefore, the protection of mitochondria function is one of the neuroprotective approaches in Parkinson's disease.

Centella asiatica (Apiaceae) is a medicinal plant widely used in South East Asia. The major active compounds of *C. asiatica* are madecassoside and asiaticoside (Günther and Wagner, 1996). Various pharmacological effects of *C. asiatica* extract have been reported, including antioxidant (Gnanapragasam et al., 2004), anti-inflammatory (Ramesh et al., 2014) and neuroprotective effects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Haleagrahara and Ponnusamy, 2010). Since the crude extracts of *C. asiatica* may vary in their compositions, contributing to the variation of pharmacological responses, ECa233, a standardized extract of *C. asiatica*, has been established (Saifah et al., 2009). ECa233 consists of madecassoside and asiaticoside in concentration of 80% or more, and the ratio between madecassoside and asiaticoside is 1.5 (± 0.5):1 (Anukunwithaya et al., 2017b; Saifah et al., 2009). ECa233 exhibited pharmacological effects in the central nervous system, including neuritogenic effects *in vitro* (Wanakhachornkrai et al., 2013), anxiolytic effects in mice (Wanasuntronwong et al., 2012) and neuroprotective effects in mice with amyloid-β-induced memory deficit (Kam-Eg et al., 2009). In this study, the neuroprotective effects and the underlying mechanisms of ECa233 have been investigated in parkinsonism rats induced by rotenone.

Materials and methods

Chemicals and reagents

The standardized extract of *Centella asiatica* ECa233 was prepared by a patent-pending method (Saifah et al., 2009) and provided by Siam Herbal Innovation Co., Ltd. (Samutprakan, Thailand). The contents of madecassoside (52%) and asiaticoside (41%) were quantified by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Rotenone, apomorphine, carboxymethylcellulose (CMC), paraformaldehyde (PFA), 1,1,3,3-tetraethoxypropane (TEP), phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and non-fat dry milk were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Loughborough, UK). Hydrogen peroxide (H_2O_2) was purchased from Ajax Finechem (Auckland, New Zealand). Rabbit monoclonal anti-SOD and anti-catalase antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-TH, anti-GAPDH, anti-goat biotin-conjugated antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies were purchased from Millipore (Billerica, MA, USA).

Animals

Male Wistar rats, 8-week old, (National Laboratory Animal Center, Mahidol University, Thailand) were housed in groups of 3 under controlled temperature (24 ± 2 °C) and relative humidity (60 ± 10%) conditions with a 12-h light/dark cycle. Food and water were provided *ad libitum*. Animal experiments were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (approval number 15-33-004).

Experimental design

Rats were divided into 5 groups (n = 10/group); control, rotenone-induced parkinsonism (PD), and rotenone-induced parkinsonism co-treated with ECa233 in concentrations of 10, 30 or 100 mg/kg (PD-ECa10, PD-ECa30 and PD-ECa100, respectively). Rats in the control and PD groups received 0.5%CMC (1 ml/kg p.o.), while rats in PD-ECa groups received various doses of ECa233 once daily for 20 days. From day 15 to 20, rotenone (2.5 mg/kg i.p.) was given to rats in PD, PD-ECa10, PD-ECa30 and PD-ECa100 groups, while rats in the control group received 2% DMSO in sunflower oil (Fig. 1).

Locomotor activity test was performed on days 1, 14, 17 and 20. Apomorphine challenge was assessed on day 21. On day 22, rats were euthanized using pentobarbital (100 mg/kg i.p.). Five rats per group were transcardially perfused with PBS followed by 4% PFA. Brains were then kept in 4% PFA for further histological investigation. Brains of the remaining rats (n = 5/group) were quickly removed and cut sagittally. Brain mitochondria were prepared from left brain hemispheres and mitochondrial complex I activity was measured. Right brain hemispheres were kept at −80 °C for further analysis of malondialdehyde (MDA) levels and superoxide dismutase (SOD) and catalase protein expression.

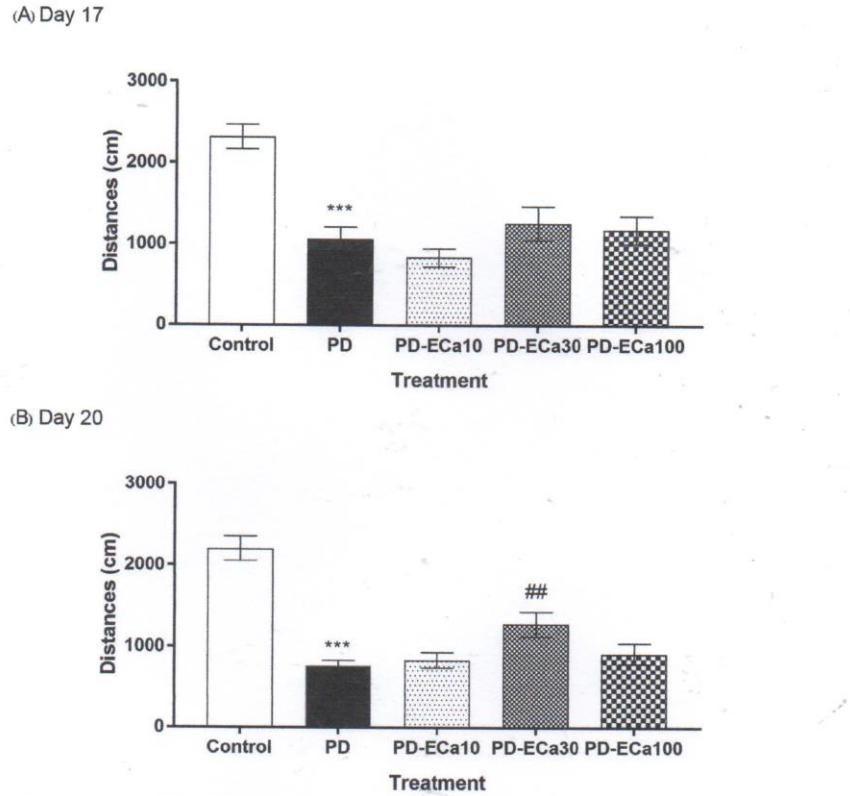

Fig. 2. Effects of rotenone and ECa233 on locomotor activity. The travelled distances on days 17 (A) and 20 (B) were presented as mean ± S.E.M. $^{***}p < 0.001$ compared to control, $^{\#\#}p < 0.01$ compared to PD.

Locomotor activity

Each rat was placed in a black square box (50 × 50 × 40 cm) for 5 min. The travelled distances were recorded and analyzed using VideoMOT2 system (TSE Systems, Germany).

Apomorphine challenge

To determine whether motor impairments induced by rotenone administration depended on dopamine depletion, apomorphine (0.5 mg/kg), a dopamine agonist, was given subcutaneously to all rats on day 21. Locomotor activity was tested 5 min after apomorphine injection.

Immunohistochemistry

Tyrosine hydroxylase (TH) immunostaining was used to determine dopaminergic neurons in the substantia nigra and striatum. Brains in 4% PFA were transferred to 30% sucrose solution for 48 h. Brains were then serially sectioned (40 μm) in the coronal plane using a Leica cryostat (LEICA, Germany) at −20 °C. Brain sections from the striatum (at +2.16, + 2.04 and + 1.92 from bregma) and substantia nigra (at −5.04, −5.20 and −5.28 from bregma) were selected according to the rat brain atlas (Paxinos and Watson, 2009). The selected sections were incubated in 0.1% H_2O_2 for 30 min, and placed in 5% skim milk for 1 h. The sections were then incubated with primary anti-TH antibody (1:1000) at 4 °C overnight followed by a 2-h incubation with secondary anti-goat biotin-conjugated antibody (1:500) at room temperature. The antibody-antigen complexes were trapped with an advidin-biotin complex (Vector, CA, USA) followed by reaction with 3,30-diaminobenzidine (Wako, Japan).

To quantity the dopaminergic neurons in the substantia nigra, total TH-positive neurons were counted. In the striatum, the intensity of TH-positive neurons was measured using ImageJ software. The values were subtracted from the corpus callosum values for background correction.

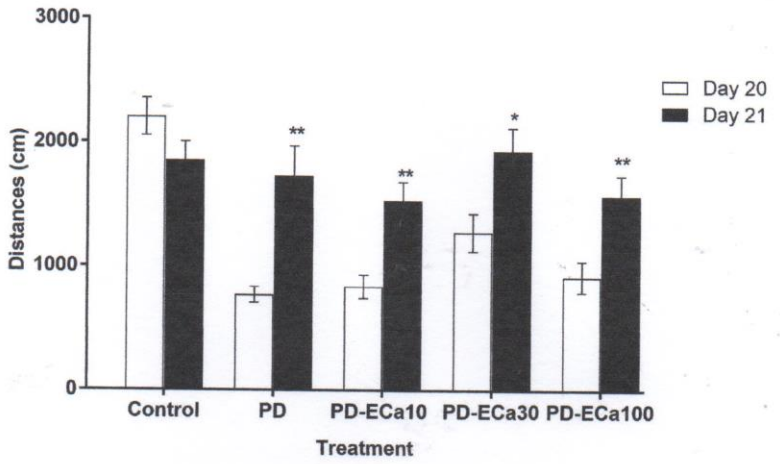

Fig. 3. Apomorphine challenge was tested on day 21. Rats received apomorphine (0.5 mg/kg s.c.). Locomotor activity was tested 5 min later. The travelled distances on days 20 and 21 were compared. *$p < 0.05$, **$p < 0.01$ compared to the same experimental group on day 20.

Mitochondrial complex I activity assay

Brain mitochondria extraction and mitochondrial complex I enzyme activity measurement were performed according to the manufacturer's instruction of Complex I enzyme activity microplate assay kit (Abcam, Cambridge, UK). The enzyme activity was measured using the kinetic mode of microplate reader (SpectraMax® M5, Molecular Devices, CA, USA) at an OD 450 nm for 30 min. The activity was normalized by mg of total protein by Bradford assay.

Lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid reactive species (TBARS) method (Euaruksakul et al., 2015). 1, 1, 3, 3-tetraethoxypropane (TEP), a MDA precursor, was used to create a standard curve.

Western blotting

The expression of SOD and catalase was determined using western blot analysis. Brain pellets were lysed in lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, sodium orthovanadate and sodium fluoride]. Total protein was determined using the BCA protein assay kit (Thermo Fisher Scientific, IL, USA). Protein samples (100 µg) were separated by 12% SDS-PAGE gel electrophoresis and electrically transferred onto a PVDF membrane. After blocking of unspecific proteins using 5% skim milk, the membranes were then incubated with the primary antibodies against SOD (1:500), catalase (1:500) and GAPDH (1:1000) at 4 °C overnight. The membranes were then incubated with HRP-conjugated secondary antibody (1:1000) at room temperature. The membranes were developed using chemiluminescence detection reagents (Thermo Fisher Scientific, IL, USA) and visualized with a luminescent image analyzer (ImageQuant™ LAS 4000, GE Healthcare Bio-sciences, Japan).

Statistical analysis

The results were expressed as the mean ± S.E.M. and analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by LSD post hoc test was used to determine the significant differences among groups. In apomorphine challenge experiment, distances were compared using a dependent *t*-test. $P < 0.05$ was considered significantly different.

Results

Locomotor activity

The locomotor activities of all rats did not differ at baseline and at 14 days after ECa233 treatment. Rotenone treatment significantly decreased the travelled distances of PD rats compared to control rats ($p < 0.001$) on days 17 and 20 (Fig. 2). Interestingly, on day 20, rats in PD-ECa30 group had travelled significantly longer distances than did PD rats ($p < 0.01$) (Fig. 2B), indicating that ECa233 (30 mg/kg) treatment can protect against rotenone-induced motor deficits.

Apomorphine challenge

Apomorphine did not alter the locomotor activity of rats in the control group but significantly improved locomotor activity of rotenone-treated rats (Fig. 3). This result indicated that motor impairment induced by rotenone resulted from presynaptic degeneration of dopaminergic neurons.

TH-positive neurons in the substantia nigra and striatum

TH-immunohistological staining was performed to illustrate the existence of dopaminergic neurons in the nigrostriatal pathway. PD rats had significantly lower numbers of TH-positive neurons in the substantia nigra than did controls ($p < 0.01$), while ECa233 (30 mg/kg) treatment significantly increased the numbers of TH-positive neurons

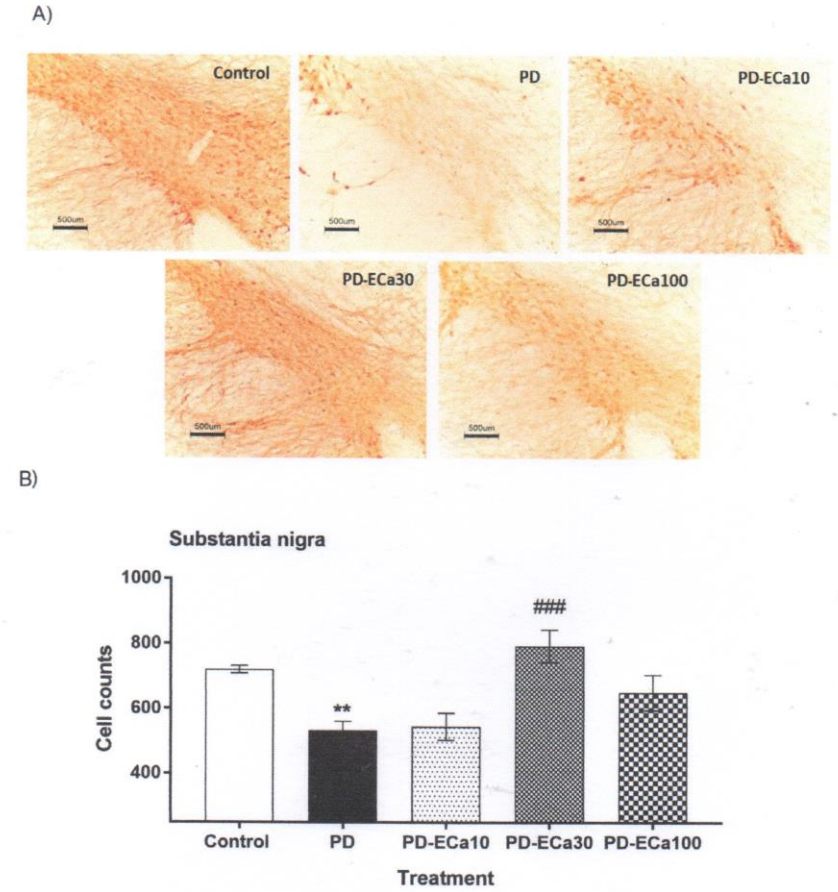

Fig. 4. Tyrosine hydroxylase immunohistostaining of dopaminergic neurons in the substantia nigra (A). The numbers of TH-positive neurons were counted and presented as mean ± S.E.M. (B). $^{**}p < 0.01$ compared to control, $^{###}p < 0.001$ compared to PD.

compared to those in PD rats ($p < 0.001$) (Fig. 4).

Similarly, striatal intensity of PD rats was significantly lower than that of the control ($p < 0.001$), while striatum intensity of PD-ECa30 and PD-ECa100 rats was significantly higher than that of PD rats ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 5).

Mitochondrial complex I activity

Mitochondrial complex I activity in the brains of PD rats was significantly reduced compared to that in control rats ($p < 0.01$). PD-ECa30 rats presented a significantly higher mitochondrial complex I enzyme activity than PD rats ($p < 0.05$) (Fig. 6) suggesting that ECa233 (30 mg/kg) can protect the inhibition of mitochondrial complex I caused by rotenone.

Lipid peroxidation

MDA levels in the brains of PD rats were significantly increased compared to the control ($p < 0.01$). In contrast, PD-ECa30 rats had lower MDA levels than PD rats ($p < 0.05$) (Fig. 7).

SOD and catalase expression

Rotenone treatment significantly reduced SOD and catalase

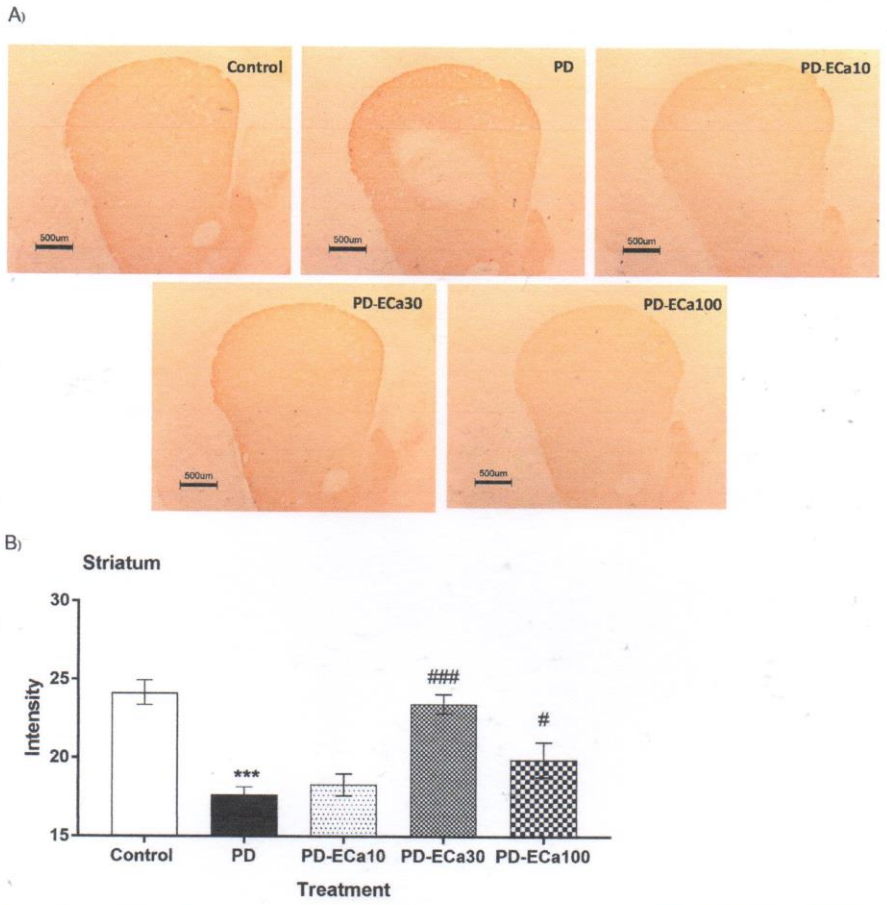

Fig. 5. Tyrosine hydroxylase immunohistostaining of dopaminergic terminals in the striatum (A). TH staining intensity was measured and presented as mean ± S.E.M. (B). $^{***}p < 0.001$ compared to control, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to PD.

expression in PD rats ($p < 0.05$). Compared to PD rats, rats treated with ECa233 (30 mg/kg) showed significantly increased SOD and catalase expression ($p < 0.01$ and $p < 0.05$, respectively). ECa233 (100 mg/kg) treatment also increased catalase expression compared to PD rats ($p < 0.05$) (Fig. 8).

Discussion

The loss of dopaminergic neurons in the nigrostriatal pathway is the main pathological hallmark of Parkinson's disease (Connolly and Lang, 2014). Mitochondrial dysfunction and oxidative stress play important roles in the pathogenesis of Parkinson's disease (Perfeito et al., 2013). Rotenone, a mitochondria complex I inhibitor, was used to induce parkinsonism behaviors and dopaminergic neuronal death in this study. The results showed that ECa233 (30 mg/kg) alleviated rotenone-induced locomotor impairment and protected against dopaminergic neuronal death. The neuroprotective mechanisms of ECa233 involved the protection of mitochondrial complex I activity, a decrease in lipid peroxidation and the elevation of antioxidant enzyme expression.

The presence of the major compositions of ECa233, madecassoside and asiaticoside, has been reported in rat brains after ECa233

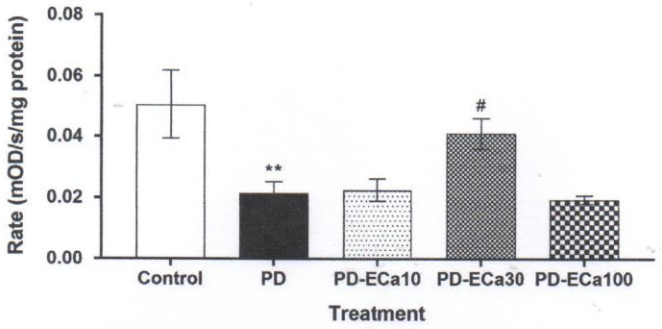

Fig. 6. Effects of rotenone and ECa233 on brain mitochondrial complex I activity. $^{**}p < 0.01$ compared to control, $^{\#}p < 0.05$ compared to PD.

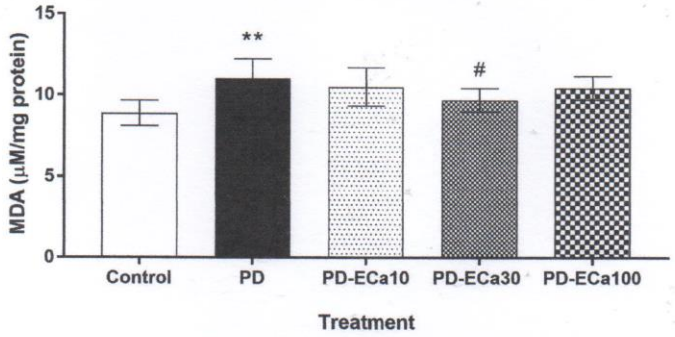

Fig. 7. Effects of rotenone and ECa233 on brain MDA levels. $^{**}p < 0.01$ compared to control, $^{\#}p < 0.05$ compared to PD.

administration (Anukunwithaya et al., 2017b; Hengjumrut et al., 2017). Previous studies demonstrated the protective effects of madecassoside and asiaticoside in rats with MPTP-induced motor deficit (Xu et al., 2012,2013). Therefore, madecassoside and asiaticoside could account for the protective effect of ECa233 in rotenone-induced parkinsonism in this study.

Dopaminergic neuroprotection of ECa233 is clearly shown in this study as the number of dopaminergic neurons in the substantia nigra and dopaminergic axon intensities in the striatum of rats in ECa233 (30 mg/kg)-treated rats was comparable to the control group (Figs. 4 and 5). It was noted that a higher dose of ECa233 (100 mg/kg) had a subtle effect in the protection of dopaminergic neurons in the substantia nigra but significantly preserved dopaminergic axon terminals in the striatum. The neuritogenic effect of ECa233 has been observed in IMR-32 neuroblastoma (Wanakhachornkrai et al., 2013). The survival of cell bodies and axonal fibers can be mediated through separate mechanisms (Ries et al., 2008). Apart from antioxidant and mitochondria protection, the ability to promote axonal regeneration is a possible neuroprotective mechanism of ECa233.

Mitochondrial complex I reduction has been reported in the substantia nigra of Parkinson's disease patients (Schapira et al., 1990). Therefore, mitochondria toxins are used to produce Parkinson's models *in vitro* and *in vivo* (Martinez and Greenamyre, 2012). *In vitro* studies showed that the active compounds in *C. asiatica*, madecassoside and asiatic acid, can protect against mitochondrial membrane potential reduction induced by H_2O_2 and rotenone, respectively (Bian et al., 2012; Nataraj et al., 2017). The current study is the first to demonstrate the protection of mitochondrial activity of the standardized extract of *Centella asiatica* ECa233 in an animal model of Parkinson's disease.

An increase in oxidative stress and a decrease in protective mechanisms contribute to cell death in Parkinson's disease (Ogunro et al., 2014). ECa233 decreased lipid peroxidation and increased antioxidant enzyme expression in rotenone models. This finding is in agreement with previous studies, which reported the antioxidative effects of *C. asiatica* extract, madecassoside and asiaticoside in MPTP models (Bhatnagar et al., 2017; Haleagrahara and Ponnusamy, 2010; Xu et al., 2012,2013). The protection of mitochondrial function possibly contributes to the reduction of oxidative stress.

Notably, the neuroprotective effects of ECa233 occurred in a dose-independent manner. ECa233 30 mg/kg markedly alleviated rotenone-induced toxicity, while the subtle effect of ECa233 100 mg/kg was presented. The roles of efflux transporters may explain this observation as madecassoside is a substrate of p-glycoprotein and multi-drug-resistant protein (Leng et al., 2013). In addition, asiatic acid, a component of *C. asiatica* extract and a metabolite of asiaticoside, induced p-glycoprotein expression *in vitro* (Abuznait et al., 2011). Recently,

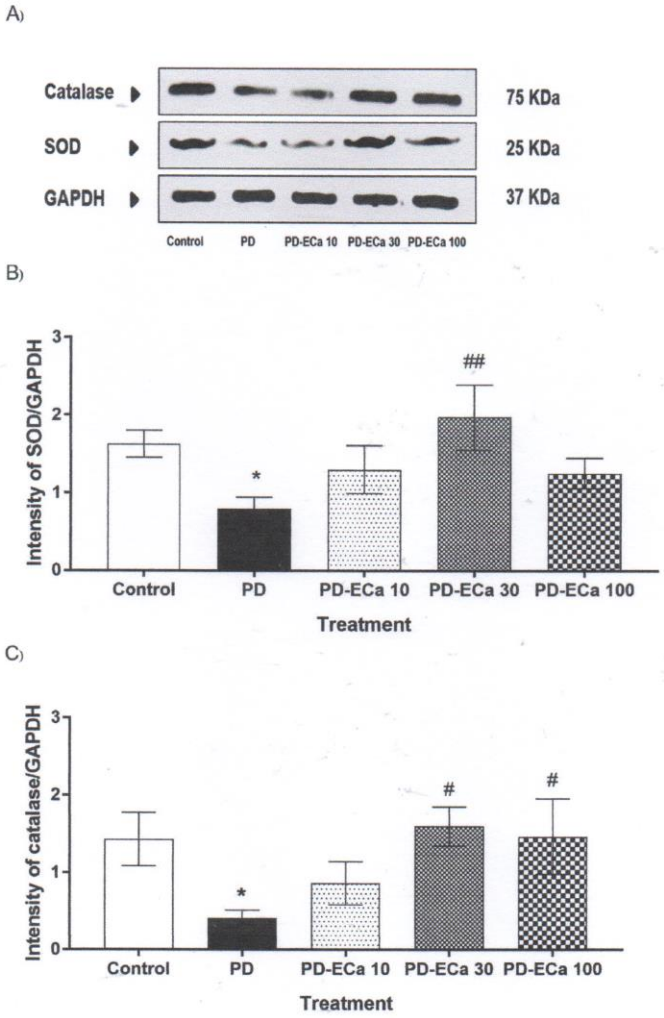

Fig. 8. Effects of rotenone and ECa233 on SOD and catalase expression (A). The levels of SOD (B) and catalase (C) expression were calculated and expressed as SOD/GAPDH and catalase/GAPDH intensity. $^{*}p < 0.05$compared to control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to PD.

Anukunwithaya et al. (2017a) reported the increases of *Abcb1a* and *Abcc2* mRNA expressions in the small intestine of rats receiving ECa233 for 7 days. Taken together, the induction of efflux transporter by ECa233 can lead to a lower effect of ECa233 at 100 mg/kg.

In conclusion, a standardized extract of *Centella asiatica* ECa233 exhibits neuroprotective effects in rotenone-induced parkinsonism rats. The mechanisms might be mediated through the prevention of mitochondrial dysfunction, the increases of antioxidant enzyme expression and the decrease of lipid peroxidation, resulting in neuroprotection and motor improvement. These findings suggest that ECa233 is a

promising candidate for Parkinson's disease treatment. However, further pharmacokinetic studies with repeated administration as well as synergistic pharmacodynamic effects of ECa233 should be conducted to fully clarify the dose-independent effects and other underlying mechanisms of ECa233.

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Conflict of interest

The authors declare that there is no conflict of interest.

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