Pharmacological study of tianma gouteng yin: a traditional Chinese medicine formula for Parkinson's disease

Liangfeng Liu
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Pharmacological Study of Tianma Gouteng Yin
– a Traditional Chinese Medicine Formula for Parkinson’s Disease

LIU Liangfeng

A thesis submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Principal Supervisor: Prof. LI Min
Hong Kong Baptist University
August 2015
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of Ph.D. at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature:

Date: August 2015
ABSTRACT

Parkinson’s disease (PD) is the second most common neurodegenerative disease affecting 2% of the population over 65 years old that lacks effective cure. The current available treatments for PD are largely symptomatic and palliative. Tianma Gouteng Yin (TGY) is a traditional Chinese medicine (TCM) formula belongs to the formulas that dispel wind. Nowadays, it has been a commonly prescribed formula to treat Parkinsonian-like symptoms such as tremor and paralysis in some of the patients. However, just as most of the TCM formula, the material basis and the underlying pharmacological effects of TGY are still lacking experimental evidence.

In this study, a method using UHPLC/Q-TOF-MS and HPLC-ELSD has been developed and successfully applied to qualitatively and quantitatively determine the complex phytochemicals of TGY. Totally 28 phytochemicals were identified, of which 20 were simultaneously quantified. The material basis profile of TGY decoction was delineated for the first time.

After full component analysis of TGY, the neuroprotective activity of TGY was verified both in vivo and in vitro. In Drosophila PD models, TGY mitigated rotenone induced toxicity and promoted α-synuclein clearance. In stereotaxic rotenone intoxication rats, TGY exerted neuroprotective effects in terms of preventing dopaminergic neurons loss and alleviating neuroinflammation. TGY alleviated rotenone induced apoptosis in SH-SY5Y cells. Furthermore, we discovered that Geniposide, an important component of TGY, is an autophagy inducer both in vivo and in vitro and is neuroprotective in
transgenic *Drosophila* PD model. In general, our study proves that TGY is neuroprotective in PD models.

In addition to the efficacy study, safety of TGY application in terms of TGY-drug interaction was also evaluated. In our study, herb-drug interactions between TGY and one of the most popular drugs used in PD treatment, Sinemet, were studied. The pharmacokinetics data showed that co-administration of TGY could suppress the absorption of Levodopa, the main component of Sinemet, for the first time. This information suggest that in clinical practice, TGY should avoid been administrated with Levodopa containing medicaments at the same time.

In conclusion, the data of this study provides valuable information on the material basis, efficacy and safety of TGY. This information is useful reference for the clinical application of TGY in PD treatment.

**Keywords:** Tianma Gouteng Yin, Parkinson’s disease, *Drosophila*, Rotenone, \(\alpha\)-synuclein, Geniposide, Autophagy, Sinemet, Herb-drug interaction
ACKNOWLEDGEMENTS

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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association studies</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>LBs</td>
<td>Lewy bodies</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>ALP</td>
<td>autophagy-lysosomal pathway</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine</td>
</tr>
<tr>
<td>MPP(^+)</td>
<td>1-methyl-4-phenylpyridinium ion</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ST</td>
<td>stereotaxical</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
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<tr>
<td>TGY</td>
<td>Tianma Gouteng Yin</td>
</tr>
<tr>
<td>TCM</td>
<td>traditional Chinese medicine</td>
</tr>
<tr>
<td>HDI</td>
<td>herb-drug interactions</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism or excretion</td>
</tr>
<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>UPLC</td>
<td>ultra high performance liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>LLOD</td>
<td>lower limit of detection</td>
</tr>
<tr>
<td>LLOQ</td>
<td>lower limit of quantification</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ion source</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>AP</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>ML</td>
<td>medial-lateral</td>
</tr>
<tr>
<td>DV</td>
<td>dorsal-ventral</td>
</tr>
<tr>
<td>APO</td>
<td>apomorphine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>MRM</td>
<td>multiple-reaction-monitoring</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full name</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N-dimethylformamide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median lethal dose</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
</tr>
<tr>
<td>3MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>p70s6k</td>
<td>p70S6 kinase</td>
</tr>
<tr>
<td>HDI</td>
<td>herb-drug interaction</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>CAN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient variation</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
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</table>
CHAPTER 1. Introduction

1.1 Parkinson’s disease

In 1817, James Parkinson described the primary characteristics of Parkinson’s disease (PD), which is the second most common neurodegenerative disease after Alzheimer’s disease (AD) \(^{[1]}\). Almost a century later, people finally discovered that the central pathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) with conspicuous deletion of dopamine in the striatum. The loss of SNpc dopaminergic neurons disturbed nigrostriatal dopaminergic pathway, which lead to the major symptoms of PD. Most of these symptoms can be relieved by oral administration of the dopamine precursor levodopa (L-3, 4-dihydroxyphenylalanine) \(^{[2]}\). However, long-term treatment with levodopa can cause involuntary movements, namely “dyskinesias”, to the PD patients. So far, all of the current treatments for PD are symptomatic. Thus, most of the researches of PD are focused on prevention of dopaminergic neuron degeneration.

1.1.1 Epidemiology

The prevalence of PD in developed countries is generally estimated at 0.3% of the whole population and approximately 1% in the senile people over 60 years of age \(^{[3]}\). The estimated median standardized incidence rate in industrialized countries is between 14 per 100,000 person-years \(^{[4]}\). In mainland China, the overall prevalence of PD over 22 years of age is about 190 per 100,000 people and the overall estimated incidence of PD is 362 per 100,000 person-years \(^{[5]}\). As per Hong Kong, in Chinese population, an estimated prevalence rate is 188 per 100,000 \(^{[6]}\) and the incidence of PD in the people over 55 years of age is 0.5% \(^{[7]}\). Some of the studies reported that prevalence of PD is
higher in men than in women, although other studies reported no gender variation \cite{8}.

The projected growth of PD in individuals over 50 from 2005 to 2030 is shown in Fig. 1-1.

Fig. 1-1 The projected growth of PD in individuals over 50 from 2005 to 2030 \cite{9}.

1.1.2 Risk factors

PD is an age-dependent, late-onset neurodegenerative disease. Numerous factors including genetic and environmental factors contribute to the onset and progress of the disease.

In the past two decades, studies on the genetics of familial PD cohorts largely improve our understandings about the underlying disease process at the molecular level. At least 15 PD linked mutations were identified and several of them have already been extensively studied. These genes include SNCA, LRRK2, MAPT, ATXN2, ATXN3, GCH1, DCTN1, VPS35, PINK1, PARK2, PARK7, ATP13A2, FBXO7, PANK2 and PLA2G6 \cite{10}. Through genome-wide association studies (GWAS), SNCA and LRRK2
were identified as risk factors, which can cause rare monogenic forms of PD [11]. SNCA-associated PD is an early-onset, rapid progress autosomal dominant inherited Parkinsonism. Three missense mutations, A53T, A30P, and E46K, were discovered. Duplications [12, 13] and triplications [14, 15] of SNCA were proved to be the cause of certain familial PD. In addition to these three well-known SNCA mutations, several novel SNCA missense mutations were discovered recently. They were H50Q [16], A53E [17] and G51D [18]. The neuropathology characteristic of patients with SNCA mutations is widespread α-synuclein deposits, which are predominantly in neurons but also appear in glial cells [19]. Mutations in LRRK2 gene (PARK8) are the most common cause of autosomal dominant PD [20]. Patients with LRRK2 mutations usually have typical features of PD with onset in middle or late onset. Pathologically, progressive neuronal loss, synuclein pathology and tau pathology could be observed [21]. Several mutations including I2020T, G2019S and G1441C have been characterized. The G2019S mutation is described most commonly that for the majority of familial cases and up to 1.6% of idiopathic PD cases.

Environmental toxins play an important role in PD pathogenesis. Specifically, the herbicide paraquat, fungicide maneb and insecticide rotenone have been studied extensively and employed to establish PD models [22]. Paraquat is supposed to enter the brain through neutral amino acid transporters and subsequently the cells in a sodium-dependent manner [23]. After entering central nervous system (CNS), paraquat acts as a redox cycling reagent and potentially causes indirect mitochondrial toxicity [24]. Maneb, which is a specific mitochondrial complex III inhibitor, could also pass the blood brain barrier to enter the CNS [25]. Rotenone is a naturally occurring complex ketone pesticide derived from the roots of Lonchocarpus species. It can rapidly pass cellular membranes
without the aid of transporters, including the blood-brain barrier. Rotenone is a strong inhibitor of mitochondrial complex I. It is reported that chronic systemic exposure to rotenone causes many features of PD, including nigrostriatal dopaminergic neurons degeneration \[^{26}\]. In addition, rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are frequently used to induce PD models. All of these toxins are oxidative stress inducers, which can subsequently cause oxidative damage to the neuron cells and finally cause Parkinsonian damage.

1.1.3 Neuropathology

One of the core pathological features of PD is dopaminergic neurons loss in SNpc. Compared with the unaffected individuals, approximately 50%-70% of the neurons have lost when death occurs \[^{27, 28}\]. PD is one of the synucleinopathies \[^{29}\]. Fibrils that constituted with α-synuclein formed insoluble polymers are deposited in the soma of neurons, composing spherical lamellated eosinophilic cytoplasmic inclusions, the Lewy bodies (LBs). α-Synuclein deposition also occurs in neuronal processes, namely Lewy neurites, and in oligodendrogial cells and astrocytes. Formation of LBs is the pathological hallmark of PD. The pathological staging of PD is based on the distribution of LBs. In the early stages, the LBs are primarily affecting the medulla oblongata/pontine tegmentum and olfactory bulb. The PD patients in the early stages behave as pre-symptomatic. During the later stages, the LBs can distribute in substantia nigra areas of the midbrain and basal forebrain. Finally, the pathological changes can reach the neocortex \[^{30}\].
1.1.4 Therapeutic strategies

Abnormal protein accumulation generally results from impaired clearance and/or augmented generation. The ubiquitin-proteasome and autophagy-lysosomal systems are the two cardinal cellular mechanisms in clearance of unwanted proteins. Impaired function of ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) systems has been unveiled in PD patients \[^{31}\]. The primary component of the aggregated proteins in PD is \(\alpha\)-synuclein. The mutations or even the duplication or triplication of wild-type \(SNCA\) could trigger the onset and progress of PD \[^{14}\]. A postulation was raised that formation of \(\alpha\)-synuclein oligomers, fibrils and aggregates impairs the UPS and ALP; further proteins accumulation and ultimately neuronal death \[^{32}\]. In addition, exposure to environmental toxins, such as rotenone, possibly exacerbates the neural toxicity of \(\alpha\)-synuclein. A simplified interplay between \(\alpha\)-synuclein and environmental toxins is depicted in Fig 1-2.
In view of violent neural toxicity of α-synuclein, therapeutic strategy that decreasing the level of α-synuclein accumulation is essential. In general, one option is to reduce the expression of α-synuclein by silencing SNCA. Another option is to up-regulate the ALP and/or UPS systems in order to increase the proteolytic degradation of α-synuclein. Moreover, those therapies, which antagonize environmental toxins, that maintaining regular function of mitochondria and eliminating oxidative stress would be in favor of PD treatment. Based on this knowledge, discovering small molecules as therapeutic agents for PD is feasible. In the previous findings of our lab, small molecules originated from the herbs of traditional Chinese medicines were able to promote ALP for the degradation of α-synuclein. The drugs that are available in the market or under

Fig. 1-2 Interplay between α-synuclein and environmental toxins in PD \cite{33,34}.
development are shown in the Fig. 1-3. Most of the market drugs for PD treatments are largely palliative and symptomatic. Therapies that are real neuroprotective to PD are still wanted.

Fig. 1-3 Existing and evolving treatments for Parkinson’s disease [36].

1.2 PD models

The discovery of Levodopa pushes forward the symptomatic treatment of PD since 1960s. However, the prognosis of PD has not been changed. Neuroprotective therapy that slows the progression of neuronal dysfunction and dopaminergic neuron loss is not yet available [37]. One of the main impediments to the progress of the development of
such therapy is lacking of sufficient appropriate animal models of the disease. In the past four decades, neurotoxins intoxicated animal models have been widely used in PD studies. These models are able to mimic certain parts of the pathological and phenotypic characters of PD. In the past 20 years, studies of mutations that bring about rare familial forms of PD declared the advent of ‘genetic era’ of PD. The endeavor invested on PD-related gene mutations expanded our understandings of this neurodegenerative disorder and further guided the investigation of neuroprotective treatment for PD. However, there is still no ‘perfect’ PD animal model since both toxic and transgenic PD animal models have their own limitations. Most of the toxic and transgenic PD models were established in murine and Drosophila melanogaster. The current available major PD animal models based on these two organisms are summarized as follows.

1.2.1 Modelling PD in murine

**Transgenic models**

Gene mutations that trigger PD in human stipulate the mechanistic connection between the transgenic models and the disease [38]. SNCA was the first reported gene linked to familial PD. Numerous α-synuclein transgenic mice or rats have been created using different promoters. The expression of two mutations of SNCA, A30P and A53T, under the rat tyrosine hydroxylase (TH) promoter, which specifically drives the expression of the genes in catecholaminergic neurons leads to moderate striatal dopamine loss and mild dopaminergic neuron loss in substantia nigra. However, no signal of α-synuclein accumulation was observed [39, 40]. The expression of truncated α-synuclein with rat TH promoter caused moderate α-synuclein aggregation in SN, mild striatal dopamine depletion and dopaminergic neuron loss in SN [41]. Some models exert ubiquitin-positive inclusions or α-synuclein aggregates that resistant to protease K in SN [42, 43].
Using mouse prion promoter to overexpress mutated α-synuclein in mice able to develop fibrillar inclusions and α-synuclein fibrils accumulations, yet not in SN dopaminergic neurons [44, 45]. Intriguingly, some of these models developed non-motor phenotypes, such as cognitive and gastrointestinal symptoms [46-48]. Rats overexpressing human A53T and A30P SNCA mutations showed no motor dysfunction, albeit olfactory deficits were observed [49]. LRRK2 is a causative gene for autosomal dominant PD. Overexpression of wild type LRRK2 in bacterial artificial chromosome mice upregulated striatal dopamine release without affecting dopamine uptake or tissue content. Nevertheless, overexpression of G2019S mutation of LRRK2 in BAC mice showed an age-related decrease of striatal dopamine level and downregulated striatal dopamine release and reuptake [50]. Expression of G2019S mutant LRRK2 induced an age-dependent of dopaminergic neurons degeneration in nigrostriatal pathway. Moreover, reduced neurites complexity and abnormalities in autophagy and mitochondria were observed in the aged G2019S mutant carrying mice [51]. PINK1 gene mutations cause the form of autosomal-recessive early-onset PD [52]. PINK1 is a mitochondrial kinase, which is able to recruit Parkin protein to bind to depolarized mitochondria that inducing mitophagy [53, 54]. PINK1 knock out (KO) mice is more susceptible to oxidative stress and reactive oxygen species production [55]. Mitochondrial dysfunction was also observed in PINK1 KO mice [55-57]. PINK1 G309D mutation transgenic mice emerged age-related dopamine reduction and locomotor defect, in which PINK1 function was completely loss [56]. PARK2 gene encodes Parkin protein. Its mutations lead to loss of function of Parkin and subsequently trigger early-onset PD. Parkin plays an important role in the mitochondria quality control [53]. PARK2 KO mice are more susceptible both to neurotoxins and inflammatory stimuli [58]. Alterations in energy metabolism, protein handling and synaptic function were also
observed in PARK2 KO mice \[^{[59]}\]. \textbf{PARK7} encodes the protein \textit{DJ-1}, a redox-sensitive chaperone protein \[^{[60, 61]}\]. Defects in \textit{PARK7} are inducements for autosomal recessive early-onset PD \[^{[62]}\]. \textit{PARK7} KO mice have mitochondrial dysfunction \[^{[63, 64]}\], but they don’t show major neuronal anomalies.

\textit{Toxic models}

Toxic models are the most conventional experimental PD models, which mainly target on reproducing the pathological and behavioral phenotypes of human PD via intoxication with neurotoxins systematically or locally. \textit{MPTP} is a neurotoxin precursor to 1-methyl-4-phenylpyridinium ion (MPP\(^{+}\)). MPTP is able to cross blood-brain barrier (BBB) and oxidized by monoamine oxidase B into its active form-MPP\(^{+}\) \[^{[65]}\]. MPP\(^{+}\) could be absorbed by SN dopaminergic neurons through dopamine transporter and then inhibiting the mitochondrial complex I activity and finally lead to death of dopaminergic neurons \[^{[66]}\]. A reliable protocol has been described for producing reliable PD model with MPTP \[^{[67]}\]. This model successfully recapitulates the key neuronal pathological feature of PD - selective loss of SN dopaminergic neurons. However, no Lewy body -like inclusions were observed in the course of SN lesion. \textit{Paraquat} is an herbicide, which shares similar structure with MPP\(^{+}\). Mice injected with paraquat showed motor dysfunction and loss of SN dopaminergic neurons in dose- and age-dependent manner \[^{[68-70]}\]. However, the toxicity mechanism of paraquat is distinct from that of MPP\(^{+}\) albeit they share similar structure. Paraquat is not a good substrate of dopamine transporter \[^{[71]}\] and its toxicity is mainly mediated by diaphorase \[^{[72]}\] that producing superoxide. Moreover, paraquat is not a mitochondrial complex I inhibitor \[^{[71]}\].

\textbf{6-Hydroxydopamine (6-OHDA)} is one of the most often used neurotoxins to induce PD model via intracerebral injection. Historically, this prototypical PD model is the first
PD model that ever been established [73]. Because of its chemical structure, 6-OHDA does not pass the BBB. Thus, local injection is needed. Once the 6-OHDA enter the neurons, it is transiently auto-oxidized and producing high level of hydrogen peroxide that inhibit mitochondrial complex I activity and finally leads to neuron death [74]. Injection of 6-OHDA into the SN or medial forebrain bundle causes high level of nigral dopaminergic neuron loss and striatal dopamine depletion [73]. Another option is to inject 6-OHDA into striatum, which induces prompt impairment of striatal terminals following progressive cell loss of SN. This procedure granted moderate impairment of dopaminergic neurons in substantia nigra [75] Rotenone is a crystalline ketonic compound used as pesticide, insecticide and fish poison [76]. Rotenone enters the cell in DAT-independent manner and cross BBB easily because of its high lipophilic character. It is a mitochondrial complex I inhibitor causing massive generation of reactive oxygen species and inhibit proteasome activity to induce proteolytic stress. More importantly, rotenone recapitulates key phenotypic feature of PD, including selective nigrostriatal dopaminergic neuron loss and LB-like inclusion, albeit a substantial variability in the individual response to the toxin when administrated systematically [26, 77]. To overcome such limitation, stereotaxical (ST) intracerebral infusion of rotenone was tested on rats. In this route of administration, similar pathological changes without peripheral toxicity or mortality and high success rate were achieved [78]. This rotenone-based ST model reproduces the gradual and specific loss of dopaminergic neurons and mimics the clinical features of idiopathic PD and was successfully employed in searching neuroprotective therapies [79, 80]
1.2.2 Modelling PD in *Drosophila melanogaster*

**Transgenic models**

In the past 15 years, *Drosophila melanogaster* has emerged as a powerful tool for studying mechanisms of neurodegenerative diseases including PD. Modelling PD in *Drosophila* brings about lots of advantages for studying molecular and cellular pathology of PD. These advantages include shorter life span of the fruit fly, large quantity of progeny, various techniques and tools to manipulate gene expression and its well-studied anatomy and phenotypes. Moreover, *Drosophila* and human genomes share a high degree of conservation in basic biological pathways. The first transgenic *Drosophila* PD model was developed using wild-type human SNCA and two familial mutant forms (A53T and A30P), for which no *Drosophila* homologue exists\[81\]. Using the GAL4/upstream activating sequence (UAS) system, overexpression of α-synuclein in the *Drosophila* brain elicited age-dependent selective loss of dopaminergic neurons, indicating by decreased TH immunostaining in dopaminergic neurons. Formation of LB-like α-synuclein was also observed, as well as motor dysfunction indicated by climbing defects of the *Drosophila*. Unlike SNCA, most of the familial PD related genes have at least one *Drosophila* homologue. *PINK1* mutations caused loss-of-function of the relative proteins that inducing dopaminergic neuron loss and flying deficits of *Drosophila*. Overexpression of *PINK1* in *Drosophila* rescued α-synuclein induced phenotypes including dopaminergic neuron loss and climbing deficiency. *PARK2* null mutant reduced life span of the *Drosophila*. Dysfunction of mitochondria and flight muscle degeneration as well as decreased proteasome activity was detected. Due to the studies performed on *Drosophila*, the pivotal role of *PARK2* and *PINK1* in regulation of mitochondrial function and physiology such as fusion/fission process was unveiled\[82-84\]. In contrast to mammals, two *DJ-1* homologues exist in *Drosophila*, *DJ-1α* and *DJ-
They exhibit different expression; DJ-1α is predominantly expressed in the male germline and DJ-1β, as in mammals, is ubiquitously expressed. DJ-1 KO Drosophila is viable, fertile and has normal lifespan. However, they are more susceptible to those environmental neurotoxins such as paraquat and rotenone. Similarly, DJ-1β mutants lead to loss-of-function of the protein and accumulation of ROS in Drosophila brain. Expression of human LRRK2, either wild type or mutant forms, or KO the LRRK2 in Drosophila has inconsistent results regarding neurodegeneration.

**Toxic models**

Two toxins have been applied on Drosophila to induce PD models. They are Rotenone and Paraquat respectively. Chronic exposure of sublethal rotenone in Drosophila is able to elicit locomotor defects and selective loss of dopaminergic neurons in the brain clusters. Paraquat exposure caused reduced lifespan in Drosophila. Locomotor defects caused by paraquat were also observed and quantified by negative geotaxis test. These two toxic Drosophila PD models share similar characters to the models established with murine and provide alternative tools for studying the mechanism of dopaminergic neurons degeneration. No reports on 6-OHDA and MPTP toxic Drosophila PD models were recorded so far.

Generally, there’s no ‘perfect’ PD model. Both toxic and transgenic models established with murine and Drosophila have their own advantages and limitations. When using these models in studying neuroprotective therapy for PD, particular considerations have to be made depends on the specific objectives. Taking for example, if a substantial and reproducible SN dopaminergic neurons loss is required when evaluating therapeutic agents targeting anti PD-related cell death, toxic models such as rotenone intoxication
model should be selected. Instead, if molecular mechanisms of PD were to be studied, transgenic models would be good options.

1.3 Application of Tianma Gouteng Yin (TGY) in PD treatment

Tianma Gouteng Yin (TGY, 天麻鉤藤飲), a famous decoction of traditional Chinese medicine (TCM), originated from the medical book, *New Concepts for the Diagnosis and Treatment of Miscellaneous Illnesses* (《中醫雜病證治新義》), compiled by the TCM physician HU Guangci [90]. It belongs to the formula that dispel wind, which is now utilized to calm liver Wind, clear Heat and promote blood flow in terms of TCM theory [91]. Nowadays, it has been a commonly prescribed formula to treat Parkinsonian-like symptoms such as tremor and paralysis in some of the patients. However, just as most of the TCM formula, the underlying pharmacology of TGY is still lacking experimental study.

1.3.1 TCM and PD

According to TCM theory, PD is categorized as tremors (震顫), numbness (麻痹). The pathogenesis of PD are deficiency of origin (本虛) and excess of superficiality (標實), which were categorized as follow,

1. Deficiency of spleen Qi (脾氣虧虛);
2. Insufficiency of Qi and blood (氣血不足);
3. Deficiency of Liver-Yin and kidney-Yin (肝腎陰虛);
4. Insufficiency of Yang (陽氣虛衰);
5. Stagnation of Qi and blood stasis (氣滯血瘀);
6. Stagnation of phlegm (痰阻經絡);
7. Liver-wind stirs up internally (肝風內動).

The following formulae are most frequently used to treat PD based on pattern discrimination.

1. Jia Wei Liu Jun Zi Tang (加味六君子湯)/Deficiency of spleen Qi;
2. Gui Pi Tang (歸脾湯)/Insufficiency of Qi and blood;
3. Yi Guan Jian (一貫煎)/Deficiency of Liver-Yin and kidney-Yin;
4. Bu Yang Huan Wu Tang (補陽還五湯)/Insufficiency of Yang;
5. Tong Qiao Huo Xue Tang (通竅活血湯)/Stagnation of Qi and blood stasis;
6. Er Chen Tang Jia Jian (二陳湯加減)/Stagnation of phlegm;
7. Tianma Gouteng Yin (天麻鈎藤飲) & Zhen Gan Xi Feng Tang (鎮肝熄風湯)/Liver-wind stirs up internally.

Obviously, TGY falls in the category of formula that treats PD symptoms caused by Liver-wind agitation according to TCM theory.

1.3.2 The compositions and rationale of TGY

The compositions of TGY [92] (one human dose) are *Gastrodiae Rhizoma* (天麻) 9g, *Uncariae Ramulus Cum Uncis* (鈎藤) 12g, *Haliotidis Concha* (石決明) 18g, *Gardeniae Fructus* (梔子) 9g, *Scutellariae Radix* (黃芩) 9g, *Cyathulae Radix* (川牛膝) 12g, *Eucommiae Cortex* (杜仲) 9g, *Leonuri Herba* (益母草) 9g, *Taxilli Herba* (桑寄生) 9g, *Polygoni Multiflori Caulis* (夜交藤) 9g, *Poria* (茯神) 9g. In this formula, according to TCM theory, *Gastrodiae Rhizoma* and *Uncariae Ramulus Cum Uncis* are chief herbs able to normalize the liver and clears Heat. *Haliotidis Concha* normalizes liver, suppresses Yang, clear Heat, which promotes the ability of the chief herbs to normalize the liver and calm liver Wind. *Cyathulae Radix* conducts blood downward and promotes
blood flow. These two work as deputy herbs. The rest seven herbs are the assistant herbs. *Gardeniae Fructus* and *Scutellariae Radix* purge Fire and clear Heat. *Leonuri Herba* invigorates blood and promotes diuresis. *Eucommiae Cortex* and *Taxilli Herba* nourish the kidney and the liver Yin. *Polygoni Multiflori Caulis* and *Poria* calm the spirit and bolster decisiveness. All 11 herbs concert to calm liver Wind, clear Heat and promote blood flow.

1.3.3 Neuroprotective components of TGY

TGY is comprised of 11 herbs. As shown in Table 1-1, some of the small molecules derived from these herbs were reported to be neuroprotective. The bioactive components of the herbs comprise the pharmacology foundation of TGY. However, the anti-PD activity of the TGY decoction is yet to be elucidated.
Table 1-1 Neuroprotective compounds derived from TGY herbs.

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Neuroprotective activity</th>
<th>Herb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Rhynchophylline</td>
<td>®neuroinflammation®[93, 94]; ®apoptosis®[93]; ®oxidative stress®[95]</td>
<td>Uncariae Ramulus Cum Uncis (钩藤)</td>
</tr>
<tr>
<td></td>
<td>Isorynchophylline</td>
<td>®neuroinflammation®[93]; ®oxidative stress®[97];</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corynoxine B</td>
<td>®α-synuclein®[100]; ®autophagy®[100]; ®calcium®[99]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isorynchophylline</td>
<td>®neuroinflammation®[93]; ®oxidative stress®[100];</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corynoxine</td>
<td>®α-synuclein®[100]; ®autophagy®[100]; ®calcium®[99]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corynoxeine</td>
<td>®calcium®[101, 102]; ®calcium®[99]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leunorine</td>
<td>®oxidative stress®[100]; ®apoptosis®[100]</td>
<td>Leonuri Herba (益母草)</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Baicalin</td>
<td>®apoptosis®[105]; ®oxidative stress®[106]; ®neuroinflammation®[106]</td>
<td>Scutellariae Radix (黄芩)</td>
</tr>
<tr>
<td></td>
<td>Baicalein</td>
<td>®apoptosis®[107]; ®oxidative stress®[108]; ®neuroinflammation®[109]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wogonin</td>
<td>®autophagy®[100]; ®neuroinflammation®[111]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oroxylin A</td>
<td>®neurogenesis®[112]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>®oxidative stress®[113]; ®apoptosis®[114];</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperoside</td>
<td>®apoptosis®[115]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rutin</td>
<td>®oxidative stress®[116]; ®neuroinflammation®[117]; ®β-amyloid oligomer®[118]</td>
<td>Eucommiae Cortex (杜仲)</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>®oxidative stress®[119]; ®dopaminergic neuron loss®[119]</td>
<td>Leonuri Herba (益母草)</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>®calcium®[120]; ®apoptosis®[121]</td>
<td>Polygoni Multiflori Caulis (首乌藤)</td>
</tr>
<tr>
<td>Phenolic</td>
<td>Gastrodin</td>
<td>®ischemic injury®[127]; ®neuroinflammation®[128]</td>
<td>Gastrodiae Rhizoma (天麻)</td>
</tr>
<tr>
<td>compound</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.3.4 Potential TGY-drug interaction.

Peter Mere Latham [129], a great medical educator, has ever said “Poisons and medicine are oftentimes the same substance given with different intents.” This saying should be kept in mind by every medical practitioner because safety is the paramount law of pharmacy.

Polypharmacy is quite common in clinical practice. Especially in Chinese community of Hong Kong, Taiwan and Mainland, some patients seek therapy from TCM and western medicine concurrently. This actuality apparently increase the probability of herb-drug interactions (HDI). Many TCM herbs and western drugs work as therapeutic and toxic at different concentration. HDI may increase the pharmacological or toxicological activities of either component. In essence, HDI involve pharmacokinetic and pharmacodynamic mechanisms. Pharmacokinetic interactions may take place during the
course of absorption, distribution, metabolism or excretion (ADME) of the herb(s) or drug(s), which are resulting from the alteration in gastrointestinal functions; induction and/or inhibition of metabolic enzymes and transport proteins; and alteration in renal excretion\textsuperscript{[130]}. Pharmacodynamic interactions may also occur due to the pharmacologic activities of the herbs and drugs. Diverse pharmacodynamic interactions could happen, which include synergistic, additive and/or antagonistic effects.

Most of the PD patients adopting TCM therapy are already taking conventional drugs such as Sinemet, and then continue to take both types of drugs at the same time. This is not unusual, it has been reported that nearly two-thirds of PD patients worldwide resort to various kinds of complementary or alternative medicine, including TCM, which may possibly influence symptoms of PD, and/or the effectiveness of dopaminergic therapy\textsuperscript{[131]}. There is no report on TGY-drug interaction so far. Nonetheless, some of the composition herbs were reported with HDI. \textit{Scutellariae Radix} was reported could inhibit human UDP-glucuronosyl transferase\textsuperscript{[132]}, which is one of the most important phase II drug metabolizing enzymes. \textit{Uncariae Ramulus Cum Uncis} was reported able to attenuate monoamine oxidase B (MAO-B) activity\textsuperscript{[133]}. Inhibition of MAO-B activity is used as treatment for PD. Genipin, an aglycone from the herb \textit{Gardeniae Fructus}, could enhance multidrug resistance-associated protein 2 mediated choleresis and organic anion transport\textsuperscript{[134]}. \textit{Leonuri Herba} potentiates antithrombotic and antiplatelet activities and increases risk of bleeding. Coma may result from the synergistic sedative effect when co-administrated with \textit{Leonuri Herba} and benzodiazepines\textsuperscript{[135]}. \textit{Polygoni Multiflori Caulis} influences the enzyme activities of CYP1A2, CYP2E1 and CYP3A4.
These evidences indicate various mechanisms through which HDI can happen when concomitant use of herbs and conventional drugs. HDI could lead to devastating therapeutic failure or even devastating side effects.

Nowadays, the first-line drugs in the treatment of PD are Levodopa containing medicaments, such as Sinemet. Sinemet is the combination of carbidopa and levodopa. It is administered orally to improve the symptoms of PD. Levodopa is converted to dopamine by DOPA decarboxylase to maintain the level of CNS. However, such conversion takes place both in CNS and in preripheral circulation. High concentration of dopamine in peripheral system could activate the peripheral dopamine receptors result in unwanted side effects such as vomiting and nausea. Carbidopa is a DOPA decarboxylase inhibitor, which is not able to cross the blood-brain-barrier and inhibit the peripheral conversion of levodopa to dopamine and thus prevents the peripheral side effects of levodopa. According to the reference[137], carbidopa reduces the amount of levodopa needed by about 75% through increasing plasma concentration and half-life of levodopa and decreasing the elimination of peripheral levodopa. The plasma half-life of levodopa is 50 min (without carbidopa) and 1.5 hrs (with carbidopa). It was reported that some herb could alter the pharmacokinetics of levodopa when concurrently administered with Sinemet[138]. For the purpose of safety and efficacy on the application of TGY and western drug, the potential HDI between TGY and Sinemet, especially in terms of pharmacokinetic interaction, deserved further in-depth study.
CHAPTER 2. Objectives of the study

TGY, a traditional Chinese herbal decoction, is used to treat symptoms are now defined as PD. However, the clinical application of TGY is largely empirical and lacking supports of scientific evidences. Whether TGY works as a palliative therapeutic like Levodopa or reacts as a neuroprotectant remains elusive. The main target of the study is to provide experimental evidences pertaining the safety and efficacy of TGY.

TGY is comprised of 11 herbs and clinically dosed in decoction. The components of the decoction are not well understood. Because of the complex nature of TGY decoction and the lack of knowledge of its active constituents, a thorough components analysis is indispensible prior to testing its safety and effectiveness. In consideration of TGY dosage form, most of the components must be hydrophilic. Thus, a compatible analysis platform such as liquid chromatography based techniques should be adopted in TGY components analysis. Hence, the first objective of the study is to determine the components of TGY decoction.

The available evidences of effectiveness of TGY in PD treatment are all based on clinical observations. In other words, the claimed effectiveness of TGY is symptom-based. This is understandable because no matter ethically or practically, performing thorough analysis on patient is not as feasible as that on animals. Whether TGY is palliative or neuroprotective is a major question that needs to be answered. Rotenone-intoxicated and α-synuclein transgenic PD models provide vigorous tools for studying the effectiveness of TGY on PD. Therefore the second objective of the study is to evaluate the neuroprotective effect of TGY on PD models.
Currently, no report on adverse effect of TGY is available. That means the current clinical application of TGY following the guidance of physician is safe. Most of the PD patients receiving TGY are taking western drugs concomitantly. Attention should be paid on such polypharmacy conditions, which possibly give raise to potential HDI. Nowadays, the first-line prescription medications for PD treatments are Levodopa containing preparations. Sinemet (Carbidopa-Levodopa) is one of the typical Levodopa based preparations. There is no complaint by the patient that receiving both of them concurrently. However, that does not mean a critical condition does not exist. Thus, the third objective of the study is to study the pharmacokinetic interaction between TGY and Sinemet.

Finally, we expect that by accomplishing these objectives, a comprehensive understanding on TGY’s pharmacological characteristics for PD treatment could be achieved.
CHAPTER 3. Components analysis of TGY

3.1 Introduction

In clinical practice, various medicinal herbs are used in different combinations, namely formula, according to TCM theory for a long history\(^\text{[139]}\). Most of the TCM formulas were prepared in the form of decoction, which were comprised of complicated mixtures of different types of phytochemicals\(^\text{[140-142]}\). However, as a result of the diversities and huge dynamic range on the content of phytochemicals in medicinal herbs that used in TCM formulas, there is a huge “black box” between the clinical practice and pharmacological studies. Hence, profiling components of TCM formulas at both qualitative and quantitative aspects turns into a key issue in TCM study.

In recent years, analytical techniques and methodologies are developed with a great advancement. Among them, ultra high performance liquid chromatography (UPLC) with mass spectrometry (MS) has emerged as a powerful tool in the field of translational medicine study including TCM\(^\text{[143, 144]}\). Advancements in UPLC realize the fast separation of compounds with wide range of polarity providing high resolution and high retention-time reproducibility. Varieties in MS techniques, such as ion trap, quadrupole time-of-flight and triple quadrupole provide a “Swiss army knife” to researchers. The advancements of these two areas concerts the primary advantages of UPLC-MS that of high sensitivity, specificity, and fast analysis method development, comparing to traditional chromatography techniques.

TGY is a classic decoction of TCM formulas. Although the clinical and pharmacological studies of TGY and its component herbs have attracted increasing
interest, its quality analysis remains unsatisfactory. TGY contains 11 herbs. As a formula, TGY contains 11 herbs. These herbs bring along an extremely complex spectrum of phytochemicals. These phytochemicals include small molecules like glycosides, quinic acids, terpenes, flavonoids, and a large amount of carbohydrates such as monosaccharides, oligosaccharides, and polysaccharides. For the small molecules, Chinese Pharmacopeia lists only Baicalin and Gastrodin as quality control (QC) markers \[145\]. Published methods only focused on analysis of only a few marker compounds, namely Geniposide, Rhynchophylline, and Isorhynchophylline using thin layer chromatography and high performance liquid chromatography \[146-148\]. Their contents are less than 2.05%, which indicates that main components of TGY remain undetermined. Being the product of decoction, TGY also contains other constituents such as macromolecules, particularly a large amount of carbohydrates including monosaccharides, oligosaccharides and polysaccharides. Published analytical methods missed this critical part. As a result, the existing quality control of TGY, certainly could not meet the requirements of quality control. Thus, developing a full component analysis as a powerful qualitative and quantitative method is urgent for pharmacological study and clinical application of this traditional Chinese decoction.

In this study, a comprehensive analytical approach was established for quantitative analysis of TGY. Ultra high-performance liquid chromatography coupled with quadrupole-tandem time-of-flight mass spectrometry were used for analysis of 17 compounds, and high performance liquid chromatography coupled with evaporative light scattering detector were used to quantitatively determine 3 major saccharides. The contents of macromolecules such as polysaccharides, protein and nucleic acid were also determined by precipitation with ethanol solution. This part of the study targets to
develop a full component analysis method to decompose the phytochemical compositions of TGY decoction.

3.2 Materials and methods

3.2.1 Reagents, chemicals and other materials

Acetonitrile (MS grade) was purchased from RCI Lab scan Ltd. (Bangkok, Thailand), analytical grade formic acid was purchased from Sigma. HPLC grade methanol and ethanol were obtained from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore MilliQ-Plus system (Millipore, Bedford, MA, USA).

Reference standards of L-phenylalanine, Catechin, Genipin-1-gentiobioside, Epicatechin, Geniposide, Leonurine, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, Cyasterone, Corynoxeine, Isorhynchophylline, Isocorynaxeine, Rhynchophylline, Baicalin, OroxylinA-7-O-glucuronide, Wogonoside, Baicalein, and Wogonin were purchased from Chengdu Preferred Biotechnology Co. Ltd. (Chengdu, China). Bacalein-6-O-beta-glucopyranoside was prepared in the laboratory of Prof. Han-Dong Sun (State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China). All reference standards were confirmed by their MS spectra before use. The purities of the reference standards were confirmed to be greater than 98% by UPLC-DAD analysis based on peak area normalization. Reference standards of fructose, glucose and sucrose were purchased from Sigma (St. Louis, USA). Their structures are shown in Fig. 3-1.

All raw materials were purchased from Hong Kong Baptist University Mr. & Mrs. Chan Hon Yin Chinese Medicine Specialty Clinic and Good Clinical Practice Centre. The
voucher specimens were deposited at the School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China.

3.2.2 Preparation of TGY extract

Dry materials of the ingredients in proportions as listed here were pulverized. Roots and rhizomes were cut into small pieces prior to being pulverized. One dose TGY for human is *Gastrodiae Rhizoma* (天麻) 9 g, *Uncariae Ramulus Cum Uncis* (钩藤) 12g, *Haliotidis Concha* (石决明) 18 g, *Gardeniae Fructus* (栀子) 9 g, *Scutellariae Radix* (黄芩) 9 g, *Cyathulae Radix* (川牛膝) 12 g, *Eucommiae Cortex* (杜仲) 9 g, *Leonuri Herba* (益母草) 9 g, *Taxilli Herba* (桑寄生) 9 g, *Polygoni Multiflori Caulis* (夜交藤) 9 g, *Poria* (茯神) 9 g. After pulverizing, *Haliotidis Concha* (石决明) was boiled 30 min and mixed with the rest of the herbs powder, which was steeped with threefold tap water (v/w) for 0.5 h, then boiled for 1 h. The powder of *Uncariae Ramulus Cum Uncis* (钩藤) was added 15 min prior to the end of boiling, and then filtered. The residue was extracted with tap water for another two times. This decocting sequence and method is strictly following the routine clinical practice. After completion of decocting, the filtrate was concentrated by rotary evaporation under vacuum in a 60 °C water bath. The concentrated extract was frozen in liquid nitrogen and finally subjected to lyophilisation under vacuum of 105×10⁻³ mbar at -40°C. Final yield was powdered and then stored at -20°C.

3.2.3 Sample solutions preparation

For small molecular analysis, TGY powder was passed through a 60-80 mesh. Then 20 mg of the powder was accurately weighed and dissolved in distilled water in a 10 ml
volumetric flask at room temperature. Then all sample solutions were filtered through 0.22 µm nylon-membrane filters (Millipore, Barcelone).

For saccharides analysis, 10 mg of TGY was accurately weighed and dissolved in 2 ml water. 8 ml ethanol was added slowly to precipitate the macromolecular. After 24 h, centrifugation (4000 × g, 15 min) was performed, and then collected the supernatant and precipitation, respectively. The supernatant was condensed to remove ethanol, and then added 1 ml ACN/water (1:1, v/v) to dissolve it. After filtration with 0.22 µM nylon-membrane filters (Millipore, Barcelone), solutions were supplied to HPLC-ELSD analysis of fructose, glucose and sucrose. The precipitate was dried and weighed, giving the macromolecule content\textsuperscript{[149]}.

### 3.2.4 Standard solutions preparation

Reference standards of L-phenylalanine, Catechin, Genipin-1-gentiobioside, Epicatechin, Geniposide, Leonurine, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, Cyasterone, Corynoxine, Isorhynchophylline, Isocorynoxine, Rhynchophylline, Baicalin, OroxylinA-7-O-glucuronide, Wogonoside, Baicalein, and Wogonin were accurately weighed and dissolved in different percentage of methanol with distilled water to prepare the stock solution. Then these stocks were mixed to prepare a mixed standard solution.

Preparation of saccharide standard solutions was refer to the previous publication\textsuperscript{[150]}. Reference markers of fructose, glucose and sucrose were accurately weighed and dissolved in ACN/water (1:1, v/v). Calibration curves were obtained by appropriate dilution of these mixed standard solutions.
3.2.5 Analytical method

Chromatographic analysis was performed on an Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, a thermostatted column compartment, an auto sampler and a DAD detector. Separations were carried out on an ACQUITY UPLC BEH C18 column (2.1 mm × 100 nm, 1.7 µm, Waters, Milford, USA) at 40 °C with mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) in a gradient elution washed. The mobile phase used a gradient program as follow: 0-3 min, 2 % B; 3-9 min, 2-12 % B; 9-24 min, 12-32 % B; 24-29 min, 32-75 % B; 29-29.1 min, 75-100 % B; 29.1-32 min, 100 % B; 32.1-35 min, 100-2% B. The flow rate was 0.4 ml/min.

An Agilent 6540 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a jet stream electrospray ion source (ESI) used to acquire MS and MS/MS data in both positive and negative polarity. Data acquisition was controlled by MassHunterB.03 software (Agilent Technologies). The operating parameters were as follow: nebulizing gas (N₂) flow rate, 8.0 L/min; nebulizing gas temperature, 300 °C; jet stream gas flow, 9 L/min; sheath gas temperature, 350 °C; nebulizer, 45 psi; capillary, 3000 V; skimmer, 65 V; Oct RFV, 600 V; and fragment voltage, 150 V. Mass spectra were recorded across the mass range at 100-1700 m/z with accurate mass measurement of all mass peaks. To calculate the content of non-saccharide small molecules included in this decoction, the peak areas shown by extracted ion chromatograms were put into calibration curves, which were conducted by plotting peak areas and corresponding concentrations.
HPLC-ELSD determination conditions of saccharides were adopted as previously described\cite{150}. Briefly, an Agilent 1100 liquid chromatograph system (Agilent Technologies, Palo Alto, USA) and Alltech 2000 evaporative light-scattering detector (Grace, Deerfield, USA) were used. Chromatographic separation was performed on an Asahipak NH2P-50 4E (4.6 × 250 mm, Shodex, Tokyo, Japan) column with column temperature maintained at 30°C. The mobile phase was consisted of water (A) and acetonitrile (B) at a flow rate of 0.8 ml/min. Elution conditions were as follow:0~16 min, 78 % B; 16-20 min, 78-62 % B; 20-30 min, 62-60 % B. The drift tube temperature of ELSD was set at 120 °C, and nitrogen flow rate was set at 3.2 L/min. And the peak areas in ELSD chromatograms were collected for calculate the concentration. Calibration curves were generated by plotting the logarithmic of peak area against the logarithmic of corresponding concentrations.

3.2.6 Method validation

After the optimum analytical conditions were established, the method for quantitative analysis was validated in terms of linearity, sensitivity, precision, accuracy and stability.

Stock solutions of mixed standards were diluted to appropriate concentrations for construction of calibration curves. At least six concentrations of each reference standard were analyzed in triplicate. The calibration curves were constructed by plotting the peak area versus concentration for each constituent. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) under current conditions were determined at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

Intra- and inter-day variations were chosen to evaluate the precision of the developed method. For intra-day variability test, six independently prepared solutions of TGY
extract were analyzed within one day. For inter-day variability testing, the same sample was examined two times for three consecutive days. Variations were expressed as relative standard deviations (RSDs) of the data. RSD (%) = (standard deviation/mean) × 100%.

**Recovery** test was performed to evaluate the accuracy of the method. Operation was described as follow, standard solution at three different concentration levels (low, medium and high) was added to the TGY samples of which the content of reference compounds were known. Then analyzed the samples in parallel in accordance with the proposed method 2.4 and triplicate experiments were carried out at each level. The spike recoveries were calculated using the following equation: *Spike recovery (%) = (total amount detected-amount original)/amount spiked×100%.*

The **stability** test was conducted by analyzing the TGY extracts over periods of 0h, 2h, 4h, 8h, 12h, and 24h. The RSDs of peak areas of each compound were taken to the measures of stability.

### 3.3 Results

#### 3.3.1 Preparation of TGY water extract

Similar to most of the TCM formulas, TGY was prepared in decoction in clinical application. Controlled quality of TGY is pivotal to its pharmacological activity and repeatability of the experiments. In order to obtain TGY with consistent quality as those used in clinical practice, TGY was extracted by water and prepared scrupulously as described in section 6.2.2. The yield of TGY powder was 20.5 ± 0.3 % calculated by
data from three batches of extractions. The preparation procedure was summarized in Fig. 3-1.

**Fig. 3-1 Preparation of TGY extract.**
3.3.2 Optimization of the chromatographic conditions

In order to obtain optimum resolution, improve sensitivity and shorten analytical time, MS experiments were performed using an LC-MS system equipped with an ESI source. A full MS scan, in the form of a total ion chromatogram (TIC), was obtained.

With regard to mass spectrometric conditions, both positive and negative ionization modes were investigated simultaneously. The positive mode had much more electrospray ion information. Therefore, the positive ionization mode was chosen for subsequent experiments.

Gradient program was also optimized. Results showed that 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) with a gradient program: 0-3 min, 2% B; 3-9 min, 2-12% B; 9-24 min, 12-32% B; 24-29 min, 32-75% B; 29-29.1 min, 75-100% B; 29.1-32 min, 100% B; 32.1-35 min, 100-2% B provided the best separation and peak shapes of all investigated compounds. Formic acid with a final concentration of 0.1% was added into the mobile phase to make the ionization of the compounds in TGY completely. Representative chromatograms of reference standards and TGY samples are shown in Fig. 3-2.
Fig. 3-2 Typical UPLC-Q-TOF chromatograms of (A) TGY extract; (B) reference standards and (C) blank solvent.
3.3.3 Identification of chemical compounds

Accurate molecular mass of the compounds were obtained, the peaks in the chromatogram of TGY were primarily identified by comparing the mass spectra with reference standards and those in the literatures summarized in Table 3-1 [151-155]. Total 28 compounds were tentatively identified. MS/MS fragments and corresponding information were obtained and summarized in Table 3-2. Total ion chromatogram (TIC) in positive mode of TGY is shown in Fig. 3-2A. For analysis of saccharides, chromatograms of monosaccharides and oligosaccharides included in TGY acquired by HPLC-ELSD are shown in Fig. 3-3. Three peaks were respectively identified as fructose, glucose and sucrose by comparing their retention times with reference standards.

By comparing retention time and MS/MS data with those reference standards, the identification of 17 non-saccharide compounds was confirmed. Specifically, peaks 1-17 were unambiguously identified as L-phenylalanine, Catechin, Genipin-1-gentiobioside, Epi-catechin, Geniposide, Leonurine, 2,3,5,4'-tetrahydroxystilbene-2-O-β-d-glucoside, Cyasterone, Corynoxetine, Isorhynchophylline, Isocorynoxetine, Rhynchophylline, Baicalin, OroxylinA-7-O-glucuronide, Wogonoside, Baicalein, and Wogonin respectively. Their chemical structures are provided in Fig. 3-4.

After comparison with the literature, peaks 18-25 were tentatively identified as Vanillin, Pinoresinol diglucoside, Chrysin-6-C-ara-8-C-glu, Chrysins-6-C-glu-8-C-ara, Quercitrin, Norwogonin-7-O-glucuronide, 5,7,8,-Trihydroxy-6-methoxyflavone-7-glucuronide, and 5,6,7,-Trihydroxy-8-methoxyflavone-7-glucuronide, respectively.
Identification of peak 18 as Vanillin was based on MS spectrometric detection that gave a [M+Na]^+ molecular ion peak at m/z 153.0546 \footnote{154}. The mass chromatographic behavior of peak 19 exhibited the MS spectra ion peak [M+Na]^+ at 705.2367 that corresponds to Pinoresinol diglucoside \footnote{151, 156}. Peaks 20 and 21 in the LC-MS chromatogram exhibited [M+H]^+ ions at m/z 549.1646 and 549.1620. Fragmentation of their MS/MS spectra showed signals at 489, 459, and 429, and the percentages of them were identical to the reported data. Thus peak 20 was tentatively identified as Chrys-6-C-ara-8-C-glu while peak 21 was recognized as Chrys-6-C-glu-8-C-ara \footnote{157}. Comparing the m/z values together with MS/MS fragments patterns with the molecular structure and MS/MS data reported in the literature \footnote{151}, peak 22 was tentatively inferred to be Quercitrin. The reason was Quercitrin has a [M+H]^+ ion at m/z 449.1178, while the Quercetin aglycone fragment at m/z 303.0486 which was presented in the MS/MS spectrum due to the loss of a 176 fragment. Positive ESI analysis of peak 23 gave a [M+H]^+ ion at m/z 447.0922 and the aglycone cation at m/z 271.0617. As a result, the peak was tentatively identified as Norwogonin-7-O-glucuronide \footnote{158}. The two isomeric compounds 24 and 25 that eluted at retention times of 18.9 and 19.8 min, respectively, exhibited [M+H]^+ at m/z 477.1065 and 447.1058. According to the MS/MS chromatogram, these compounds produce ions at m/z 301. The loss of a 176 fragment in the MS/MS indicated the presence of a glucuronide. These compounds were thus tentatively identified based on their accurate mass and MS/MS spectra as 5,7,8,-Trihydroxy-6-methoxyflavone-7-glucuronide and 5,6,7,-Trihydroxy-8-methoxyflavone-7-glucuronide, respectively \footnote{158}. 


Table 3-1 List of phytochemicals (mw < 1,000) of the 11 herbs comprising TGY.

<table>
<thead>
<tr>
<th>Molecular formula</th>
<th>Name of the phytochemical</th>
<th>Calculated mass</th>
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<tr>
<td>C5H6O2</td>
<td>3-Furanmethanol</td>
<td>98.10</td>
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<td>C4H6O4</td>
<td>Succinic acid</td>
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<td>C7H8O2</td>
<td>4-Hydroxybenzyl alcohol</td>
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<td>p-Hydroxybenzaldehyde</td>
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<td>C5H5N5</td>
<td>Adenine</td>
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<td>C7H6O3</td>
<td>Protocatechualdehyde</td>
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<td>C4H4N4O2</td>
<td>Uracil</td>
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<td>C10H10O</td>
<td>4-Phenyl-3-buten-2-one</td>
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<td>Vanillin</td>
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Table 3-1 (Continued)
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</tr>
<tr>
<td>C14H20O9</td>
<td>1,2,3,5-Benzetetrol; 1,3-Di-Me ether, 5-O-beta-glucopyranoside</td>
<td>332.30</td>
</tr>
<tr>
<td>C20H30O4</td>
<td>15-Hydroxy-9-oxo-5,8(12),13-prostatrienoic acid</td>
<td>334.45</td>
</tr>
<tr>
<td>C21H20O4</td>
<td>4-Hydroxybenzyl alcohol; Bis-O-(4-hydroxybenzyl) ether</td>
<td>336.38</td>
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<td>C21H20O4</td>
<td>Gasrol</td>
<td>336.38</td>
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<tr>
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<td>Quercetin dihydrate</td>
<td>338.27</td>
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<tr>
<td>C15H14O9</td>
<td>Quercetin dihydrate</td>
<td>338.27</td>
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<td>C12H22O11</td>
<td>D(+)-Sucrose</td>
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<tr>
<td>C18H16O7</td>
<td>2',3',5,7,8-Pentahydroxyflavone</td>
<td>344.31</td>
</tr>
<tr>
<td>C15H22O9</td>
<td>Aucubin</td>
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Table 3-1 (Continued)
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<th>Calculated mass</th>
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<tbody>
<tr>
<td>C18H18O7&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2,3-Dihydro-2-(3,4-dihydroxyphenyl)-7-hydroxy-3-(hydroxymethyl)-5-benzo furan carboxylic acid</td>
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<tr>
<td>C16H26O8</td>
<td>4-Hydroxy-2,6,6-trimethyl-1-cyclohexene carboxylic acid; O-beta-D-Glucopyranoside</td>
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<tr>
<td>C21H34O4</td>
<td>Pregn-7-ene-3,11,15,20-tetrol</td>
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<td>C20H32O5</td>
<td>11,15-Dihydroxy-9-oxo-5,13-prostadienoic acid</td>
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<tr>
<td>C16H18O9</td>
<td>Chlorogenic acid</td>
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<tr>
<td>C20H22N2O4</td>
<td>Mitraphylline</td>
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<td>C18H16O8</td>
<td>2',5,5',6,7,8-hexahydroxyflavone</td>
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<td>Corynantheine</td>
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<td>C21H24N2O4</td>
<td>18,19-dehydrocorynoxinic acid</td>
<td>368.43</td>
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<td>18,19-dehydro corynoxinic acid B</td>
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<td>C17H19N5O5</td>
<td>Adenosine; 6-N-(4-Hydroxybenzyl)</td>
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<td>Skullcap flavon</td>
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<td>2',5,6,6',7,8-hexahydroxy flavone</td>
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<td>Vincoside</td>
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<td>Corynoline</td>
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<td>Corynoline B</td>
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<td>Rhynchophylline</td>
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<td>Genipin-1-gentiobioside</td>
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<td>11-Hydroxy-4-guaien-11-O-beta-D-Glucopyranoside</td>
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Table 3-1 (Continued)
<table>
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<tr>
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<th>Calculated mass</th>
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<tbody>
<tr>
<td>C23H30N2O4</td>
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<td>C21H20O8</td>
<td>5,6,7-Trihydroxy-4-(3,4,5-trihydroxyphenyl)-2-naphthalenecarboxylic acid; 3',5',6,7-Tetra-Me ether</td>
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<td>C18H26O10</td>
<td>Benzyl alcohol; O-[beta-D-Apiofuranosyl-(1-&gt;2)-beta-D-glucopyranoside]</td>
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<td>C21H22O8</td>
<td>3',4',5,6,7,8-Hexamethoxyflavone</td>
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<td>Shanzhiside methyl ester</td>
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<td>C18H22O11</td>
<td>Eucomoside A</td>
<td>414.36</td>
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<td>C29H50O</td>
<td>6'Sitosterol</td>
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<td>C21H20O9</td>
<td>6-Glucosyl-5,7-dihydroxyflavone</td>
<td>416.38</td>
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<td>8-Glucosyl-5,7-dihydroxyflavone</td>
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<td>C30H50O</td>
<td>Ulmoprenol</td>
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<td>3-Hydroxy-27-nor-28,13-oleananolate</td>
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<td>Baicalin</td>
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<td>5,7-Dihydroxy-8-methoxyflavone; 5-O-beta-D-Glucopyranoside</td>
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<td>C21H20O11</td>
<td>Astragalin</td>
<td>448.38</td>
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<td>C19H28O12</td>
<td>Acylsucroses; 6-O-(4-Hydroxybenzyl)</td>
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<tr>
<td>C30H44O3</td>
<td>3-Hydroxy-5,12,18-ursatrien-28-oic acid</td>
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<tr>
<td>C30H46O3</td>
<td>3-Hydroxylanosta-7,9(11),24-trien-21-oic acid</td>
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Table 3-1 (Continued)
<table>
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<tr>
<th>Molecular formula</th>
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<th>Calculated mass</th>
</tr>
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<tbody>
<tr>
<td>C30H44O4</td>
<td>3,4-Secolanosta-4(28),7,9(11),24-tetraene-3,26-dioic acid; (24Z)-form</td>
<td>468.67</td>
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<tr>
<td>C29H42O5</td>
<td>11,12-Epoxy-2,3-dihydroxy-24-nor-4(23)-ursen-28,13-oxide</td>
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<tr>
<td>C30H46O4</td>
<td>3,19-Dihydroxy-5,12-ursadien-28-oic acid</td>
<td>470.68</td>
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<td>3,6-Dihydroxy-12,18-ursadien-28-oic acid</td>
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<tr>
<td>C30H46O4</td>
<td>Cycloart-24-ene-3,23,28-triol; (3beta,23R)-form, 28-Carboxylic acid, 23-ketone</td>
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</tr>
<tr>
<td>C30H48O4</td>
<td>3,16-Dihydroxylanosta-8,24-dien-21-oic acid</td>
<td>472.70</td>
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<tr>
<td>C31H46O4</td>
<td>Poricoic acid C</td>
<td>482.69</td>
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<tr>
<td>C30H44O5</td>
<td>Poricoic acid B</td>
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<td>3,16-Dihydroxy-24-methylenelanosta-7,9(11)-dien-21-oic acid</td>
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<td>16-Hydroxy-3,4-secolanosta-4(28),8,24-triene-3,21-dioic acid</td>
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<td>3,16-Dihydroxy-24-oxolanosta-7,9(11)-dien-21-oic acid</td>
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<tr>
<td>C30H46O5</td>
<td>3,16,26-Trihydroxylanosta-7,9(11),24-trien-21-oic acid</td>
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<td>12-Oleanene-3,6,23,28-tetrol-28-Carboxylic acid</td>
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<td>C23H24O12</td>
<td>7,5,6,7-pentahydroxyflavone</td>
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<td>3,16-Dihydroxy-24-methylenelanosta-7,9(11)-dien-21-oic acid</td>
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<tr>
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<td>3,15,16-Trihydroxy-24-methylene-28-oic acid</td>
<td>500.71</td>
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<td>3,16,25-Trihydroxy-24-methylene-28-oic acid</td>
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<td>C21H32O14</td>
<td>Aucubigenin; 1-O-[beta-D-Glucopyranosyl-(1-&gt;6)-beta-D-glucopyranoside]</td>
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Table 3-1 (Continued)
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<tbody>
<tr>
<td>C31H46O6</td>
<td>3,16-Dihydroxy-24-methylhexose-8-methyl-26-oic acid</td>
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Table 3-2 Chromatogram and mass spectrum data (positive mode) of constituents identified using UPLC-Q-TOF-MS.

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<th>R.T. (min)</th>
<th>Observed Mass (m/z)</th>
<th>Calculated Mass (m/z)</th>
<th>Error (ppm)</th>
<th>Fragment ions (m/z)</th>
<th>Molecular Formula</th>
<th>Identification</th>
<th>Herbal Origin</th>
<th>Confirmed with reference standard</th>
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<td>2.80</td>
<td>166.0871</td>
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<td>120.0792 [M+COOH]</td>
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<td>L-Phenylalanine</td>
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<td>2</td>
<td>7.50</td>
<td>291.0862</td>
<td>5.3</td>
<td>139.0396 [M+H$_2$CO$_3$] , 243.0434 [M+H$_2$CO$_3$]</td>
<td>C$_8$H$_6$NO$_3$</td>
<td>Catechin</td>
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<td>4</td>
<td>9.30</td>
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<td>259.0656 [M+H$_2$-malose-OH]</td>
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<td>Epi-catechin</td>
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<td>181.0498 [M+H$_2$C$_4$H$_4$O$_4$] , 132.1125 [M+H$_2$C$_4$H$_4$O$_4$]</td>
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<td>OxylinA-7-O-glucuronide</td>
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<td>271.0601 [M+H$_2$-glucuronide]</td>
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<td>Baicalen-6-O-beta-glucopyranoside</td>
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<td>20</td>
<td>12.2</td>
<td>549.1646</td>
<td>5.5</td>
<td>531.1493 [M+H$_2$O]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>Chrysin-6-Cara-8-C-glu</td>
<td>S. baicalensis</td>
<td>Not yet</td>
</tr>
<tr>
<td>21</td>
<td>13.1</td>
<td>549.1620</td>
<td>0.7</td>
<td>531.1482 [M+H$_2$O]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>Chrysin-6-C-glu-8-C-glu</td>
<td>S. baicalensis</td>
<td>Not yet</td>
</tr>
<tr>
<td>22</td>
<td>14.0</td>
<td>449.1143</td>
<td>5.4</td>
<td>303.0486 [M+H$_2$-amhose]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>Quercetin</td>
<td>L. japonicus</td>
<td>Not yet</td>
</tr>
<tr>
<td>23</td>
<td>18.5</td>
<td>447.0946</td>
<td>5.4</td>
<td>271.0617 [M+H$_2$-glucuronide]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>Norwogonin-7-O-glucuronide</td>
<td>S. baicalensis</td>
<td>Not yet</td>
</tr>
<tr>
<td>24</td>
<td>18.9</td>
<td>477.1065</td>
<td>4.9</td>
<td>301.0719 [M+H$_2$-glucuronide]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>5,7,8-Trihydroxy-6-methoxyflavone-7-glucuronide</td>
<td>S. baicalensis</td>
<td>Not yet</td>
</tr>
<tr>
<td>25</td>
<td>19.8</td>
<td>477.1058</td>
<td>3.7</td>
<td>301.0718 [M+H$_2$-glucuronide]</td>
<td>C$_9$H$_6$O$_3$</td>
<td>5,7,8-Trihydroxy-6-methoxyflavone-7-glucuronide</td>
<td>S. baicalensis</td>
<td>Not yet</td>
</tr>
</tbody>
</table>
Fig. 3-3 Typical HPLC-ELSD chromatograms of (A) TGY sample and (B) reference standards (a: Fructose, b: Glucose, c: Sucrose).
**Fig. 3-4** Chemical structures of identified phytochemicals of TGY decoction. The numbers in the brackets correspond to the data of Table 3-2.
3.3.4 Method validation

Linearity calibration curves were constructed using at least six different concentrations of the reference standards. Each concentration of the reference standards was analyzed in duplicate. The data implied good correlation between concentrations and peak areas of these compounds, which were indicated by the coefficients ($r^2>0.999$). Linear ranges of 17 compounds are summarized in Table 3-3. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were measured on the basis of signal to noise ratios at 3:1 and 10:1, and the values are also listed in Table 3-3.

To evaluate the precision and accuracy of this method, TGY sample solution was analyzed under the selected condition six times within 24 hours for intra-day variation and on three successive days for inter-day variation. The RSDs of intra- and inter-day variation is 4.06% and 5.54%, which indicates the established method had satisfactory precision and repeatability.

In order to determine the accuracy of this method, experiment of spike recovery was performed. Three different concentrations (approximately equivalent to 0.8, 1.0 and 1.2 times of the matrix concentration) of reference standards were spiked into the TGY sample, repeated in triplicate, then extracted and quantified as described before. Results showed that the developed method was satisfactory with a mean recovery range from 94.37 to 105.99 and RSDs less than 4.5% for 17 positively identified compounds. As to stability analysis, the RSDs of the 17 compounds’ peaks were detected within 24h, which were lower than 4.6 %. All of the results demonstrated that the developed UPLC-Q-TOF method was sufficiently reliable and accurate for simultaneous quantification of the 17 positively identified non-saccharide compounds in TGY.
Table 3-3 Results of methodology validation in terms of linear regression, LLOD, LLOQ, repeatability, accuracy, and stability

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity range (ng/mL)</th>
<th>Equation</th>
<th>R²</th>
<th>LLOD (ng/mL)</th>
<th>LLOQ (ng/mL)</th>
<th>Repeatability (RSD%, n=6)</th>
<th>Spike recovery (RSD%, n=3)</th>
<th>Stability (RSD%, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-phenylalanine</td>
<td>50.60-404.80</td>
<td>Y = 148.2X - 704.4</td>
<td>0.9996</td>
<td>13.67</td>
<td>45.58</td>
<td>3.45</td>
<td>4.81</td>
<td>96.57(2.08)</td>
</tr>
<tr>
<td>Catechin</td>
<td>397.50-3180.00</td>
<td>Y = 33.8X - 721.8</td>
<td>0.9989</td>
<td>104.61</td>
<td>348.68</td>
<td>2.93</td>
<td>4.84</td>
<td>98.86(2.16)</td>
</tr>
<tr>
<td>Genipin 1-beta-gentiobioside</td>
<td>329.10-10530.00</td>
<td>Y = 56.9X + 10454.0</td>
<td>0.9977</td>
<td>43.38</td>
<td>144.60</td>
<td>1.20</td>
<td>3.37</td>
<td>101.88(2.21)</td>
</tr>
<tr>
<td>Epi-catechin</td>
<td>3187.50-25500.00</td>
<td>Y = 7.3X + 2059.8</td>
<td>0.9997</td>
<td>233.80</td>
<td>779.34</td>
<td>2.53</td>
<td>5.54</td>
<td>96.96(2.22)</td>
</tr>
<tr>
<td>Geniposide</td>
<td>1659.40-53100.00</td>
<td>Y = 16.9X + 18376.0</td>
<td>0.9989</td>
<td>174.67</td>
<td>582.64</td>
<td>4.06</td>
<td>4.64</td>
<td>102.71(1.99)</td>
</tr>
<tr>
<td>Leonurine</td>
<td>59.48-237.93</td>
<td>Y = 120.4X + 9810.8</td>
<td>0.9992</td>
<td>11.65</td>
<td>38.84</td>
<td>3.78</td>
<td>4.66</td>
<td>101.38(5.10)</td>
</tr>
<tr>
<td>2,3,4’-tetrahydroxystilbene-2-O-beta-glycoside</td>
<td>126.28-5387.76</td>
<td>Y = 493.9X + 11718.0</td>
<td>0.9999</td>
<td>25.25</td>
<td>84.18</td>
<td>2.58</td>
<td>4.17</td>
<td>97.14(2.19)</td>
</tr>
<tr>
<td>Cysteron</td>
<td>116.50-1864.00</td>
<td>Y = 170.7X + 5289.2</td>
<td>0.9996</td>
<td>17.83</td>
<td>59.44</td>
<td>1.05</td>
<td>4.54</td>
<td>96.77(0.99)</td>
</tr>
<tr>
<td>Corynoxide</td>
<td>13.02-208.33</td>
<td>Y = 378.9X + 1346.2</td>
<td>0.9994</td>
<td>2.77</td>
<td>9.24</td>
<td>1.55</td>
<td>3.66</td>
<td>101.45(4.45)</td>
</tr>
<tr>
<td>Isorhynchophylline</td>
<td>13.27-106.12</td>
<td>Y = 12974.0X - 33626.0</td>
<td>0.9994</td>
<td>2.14</td>
<td>7.13</td>
<td>1.19</td>
<td>4.71</td>
<td>101.76(2.68)</td>
</tr>
<tr>
<td>Isoerynixoxide</td>
<td>52.95-211.80</td>
<td>Y = 592.1X + 6742.6</td>
<td>0.9993</td>
<td>9.57</td>
<td>31.05</td>
<td>2.42</td>
<td>3.97</td>
<td>102.07(1.53)</td>
</tr>
<tr>
<td>Baicalin</td>
<td>1273.44-81500.00</td>
<td>Y = 440.0X + 6221.0</td>
<td>0.9996</td>
<td>49.97</td>
<td>166.57</td>
<td>2.50</td>
<td>4.59</td>
<td>100.78(2.65)</td>
</tr>
<tr>
<td>Rynchophylline</td>
<td>33.04-198.21</td>
<td>Y = 2767.1X - 52144.0</td>
<td>0.9988</td>
<td>7.62</td>
<td>25.41</td>
<td>3.46</td>
<td>4.43</td>
<td>95.89(0.71)</td>
</tr>
<tr>
<td>Oroxylin A-7-O-glucuronide</td>
<td>204.10-13062.50</td>
<td>Y = 132.0X + 17621.0</td>
<td>0.9992</td>
<td>28.98</td>
<td>96.62</td>
<td>1.69</td>
<td>4.89</td>
<td>97.72(3.65)</td>
</tr>
<tr>
<td>Baicalin-6-O-beta-glucopyranoside</td>
<td>259.38-4150.00</td>
<td>Y = 47.4X - 1173.4</td>
<td>0.9989</td>
<td>71.38</td>
<td>237.95</td>
<td>2.64</td>
<td>3.89</td>
<td>102.64(2.18)</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>1767.19-28275.00</td>
<td>Y = 117.5X + 127121.0</td>
<td>0.9989</td>
<td>26.61</td>
<td>88.71</td>
<td>1.89</td>
<td>4.29</td>
<td>97.29(2.79)</td>
</tr>
<tr>
<td>Wogonin</td>
<td>118.64-2548.47</td>
<td>Y = 1291.1X - 78900.0</td>
<td>0.9998</td>
<td>27.23</td>
<td>90.76</td>
<td>1.07</td>
<td>1.69</td>
<td>96.64(1.39)</td>
</tr>
</tbody>
</table>
3.3.5 Quantification of compounds in TGY

The developed UPLC-Q-TOF and HPLC-ELSD method was successfully employed to simultaneously determine the phytochemicals in TGY extract. The results are summarized in Fig. 3-5. Among these 17 non-saccharides, Baicalin, Geniposide, Wogonoside, Epi-catechin, and Oroxylin A-7-O-glucuronide were the five most abundant ones. The contents of the alkaloids, including Rhynchophylline, Isorhynchophylline, Corynoseine, Isocorynoseine that were considered bioactive constituents of Uncariae Ramulus cum Uncis (鉤藤), are few. Besides, the contents of fructose, glucose and sucrose were of considerable amount in the TGY extract. The macro-molecules were the most abundant quantified phytochemicals in TGY extract.
**Fig. 3-5** Contents of the phytochemicals in TGY extract. This figure illustrated the compositions of 1 g TGY extract. The amounts of quantified phytochemicals were given with the unit: mg/g.
3.4 Conclusion and discussion

In this part of the study, a method using UHPLC/Q-TOF-MS and HPLC-ELSD has been developed and successfully applied to qualitatively and quantitatively determine the complex phytochemicals of TGY. Totally 28 phytochemicals were identified, of which 20 were simultaneously quantified. Saccharides were also identified and quantified. The developed method provides a way to identify and quantify much greater percentage of phytochemicals of TGY decoction. The established method contributes to upgrading the quality control of TGY. Moreover, the result of the study provides a better understanding of the material basis of TGY.

It’s perceived that among the quantified non-saccharide compounds, Baicalin, Geniposide, Wogonoside, Epi-catechin, and Oroxylin A-7-O-glucuronide were most abundant. It’s not surprising, because TGY is prepared in the form of decoction and most of the flavonoids are of high polarity that makes them easily to be extracted by water. Nonetheless, high contents of flavonoids occur practically in most of the herbs [159]. In TGY, Baicalin is derived from Scutellariae Radix. Baicalin is one the most well studied phytochemical that reported with broad spectrum of bioactivities. Baicalin was reported to be neuroprotective on PD models induced by neurotoxins [160, 161] and could suppress iron accumulation [162, 163]. The aglycone of Baicalin could inhibit fibrillation of α-synuclein, disaggregate existing fibrils [164], and stabilize oligomer [165] while inhibit its formation [166]. Geniposide is an iridoid, which is well known for its antioxidative activity [167, 168]. Some reports described its neuroprotective activities on AD model [169]. Wogonoside and its aglycone have anti-oxidative effect [170], are able to inhibit microglial activation [171] and induce autophagy via influencing MAPK-mTOR pathway [172]. Epi-catechin prevents stroke damage [173] and amyloid β protein induced
neurotoxicity by inhibition of cytosolic calcium elevation \cite{174}. All of these neuroprotective phytochemicals constitute the material basis of the anti-PD pharmacological activity of TGY.

According to the result, nearly 50\% of the TGY extract remains unquantified. The major cause is that lacking of commercialized reference compounds. It is critical and essential for studying the phytochemicals of TCM formula. Most of the commercialized reference compounds are the ones easy to prepared from herbs or synthesized. Besides, most of the TCM formulas are dosed as decoction. Certain kinds of reactions amongst the phytochemicals of different herbs may take place and increase the difficulty in component analysis.

In conclusion, this part of the study enables the component recognition of TGY. The acquired knowledge of the phytochemicals compositions of TGY extract, avail us of designing the following pharmacodynamics and pharmacokinetics study.
CHAPTER 4. TGY is neuroprotective in PD models

4.1 Introduction

PD is the second most detrimental neurodegenerative disease in humans [8, 91]. According to TCM theory, internally stirring liver-wind brings the main symptoms such as shaking, tremors, or convulsion [175]. These symptoms are also observed in PD patients, namely motor symptoms [176]. TCM have been applied to treat symptoms related to PD with a long history. However, the clinical application of TCM formulas is largely empirical. TGY is one of the most common prescribed TCM formulas for PD treatment. Currently, most of the available therapeutics for PD is palliative and symptomatic [177]. The neuroprotective therapy for PD treatment is not yet available. Whether TGY is a symptomatic treatment or neuroprotective treatment is significant.

To study the neuroprotective activities of TGY, selection of appropriate animal models is pivotal. Based on the current progress in PD pathogenesis research, both environmental toxins-intoxicated and transgenic PD models were developed [178]. Among these models, the fruit fly Drosophila melanogaster has emerged as an apt model organism for studying PD-related neurodegeneration. Both environmental toxins models and transgenic models have been successfully established in Drosophila to replicate key pathological features of PD [179, 180] and have been successfully applied in PD therapeutic research [181]. Thus, Drosophila PD models were used in the study. However, in most of the pharmacodynamics studies, using only one species is not enough and not convincing. Currently, the transgenic murine PD models are not ideal since none of them recapitulates all of the features of PD, moreover, only a few models develop mild dopaminergic neurons degeneration [182]. Thus, the classic toxic murine PD
models were considered. Amongst these models, rotenone intoxicated PD model replicates many aspects of the pathology of human PD and can be used to test the protective effect of the therapeutic on dopaminergic neuron loss [78-80]. Therefore, a rotenone stereotaxic intoxication induced PD rat model was adopted.

In this part of the study, rotenone-intoxicated rats with rotenone-intoxicated and α-synuclein overexpressed Drosophila were employed as in vivo PD models to study the anti-PD activity of TGY. The underlying neuroprotective mechanism was also analyzed in vitro on SH-SY5Y cells. The expected outcome of the study could provide experimental evidences to support TGY’s clinical application in the treatment of PD.

4.2 Materials and methods

4.2.1 Reagents and antibodies

Rotenone (R8875), sodium dodecyl sulfate (L3771), N, N-dimethylformamide (D4551) and dopamine hydrochloride (H8502) were purchased from Sigma-Aldrich. Goat anti-mouse (115-035-003) and goat anti-rabbit (111-035-003) antibodies were purchased from Jackson ImmunoResearch Laboratories, INC. Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (A-11008), Alexa Fluor 594 conjugated goat anti-rabbit secondary antibody (A-11037) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M-6494) were purchased from Thermo Fisher Scientific Inc. 3,3’-diaminobenzidine (DAB) peroxidase substrate kit and VECTASTAIN Elite ABC Kit (Rabbit IgG) were purchased from VECTOR. Anti-tyrosine hydroxylase antibody (anti-TH, AB152) was purchased from Merck Millipore. Anti-α-synuclein (610787) was purchased from BD Transduction Laboratories. Anti-β-
actin (sc-47778) was purchased from Santa Cruz Biotechnology. Anti-Iba-1 (019-19741) was purchase from Wako.

### 4.2.2 Preparation of drug-containing fly food

TGY and/or rotenone was dissolved in water and mixed thoroughly with rehydrated instant *Drosophila* medium (Carolina, USA). The medium was renewed every 3 days.

### 4.2.3 *Drosophila* culture and strains

Fly stocks were raised at 25°C on standard cornmeal medium, under 50%~70% relative humidity with 12 hrs dark-light cycle. The following *Drosophila* strains were obtained from Bloomington *Drosophila* Stock Center and used in the study: *w1118* as wild type; *elav-GAL4*, which drive transgene expression in all neurons; *Ddc-GAL4* that drive transgene expression in dopaminergic neurons; and *UAS-SNCA* that carrying the wild type human *SNCA* gene.

### 4.2.4 Survival assay

*w1118* flies emerged within three days were collected and aged for three days in standard cornmeal. The flies (100 flies per group) were then transferred to vials containing freshly prepared instant medium (Carolina, Formula 4-24) and dosed as indicated every three days (The selected doses are based on the result of pilot study). The number of dead flies was counted every day. The percentage of flies that remained alive at the end of the experiment was calculated based on the starting number of flies for each treatment group.
4.2.5 Climbing assay
Climbing assay was performed as described [183] with minor modification. Briefly, groups of ten flies were transferred into 18 cm transparent plastic tubes 1hr prior to the assay for environmental acclimatization. A finishing line at 10cm from the bottom of the tubes was marked. The tubes were tapped to send flies to the bottoms of the tubes. The climbing time was recorded when at least five flies had passed the finishing line. When flies took more than 50s to climb above the finishing line, the climbing time was recorded as 50s. Five trials were performed for each group. The experiment was repeated at least three times. The mean climbing times were calculated and plotted as climbing scores.

4.2.6 Immunohistochemical analysis of Drosophila samples
Whole brain staining was performed as reported with minor modification [184]. Flies were anesthetized under CO₂ and then decapitated with forceps. Heads were transferred to 1.5 ml microcentrifuge tubes containing fixative (4% paraformaldehyde, 0.3% Triton X-100). Tubes stayed on ice for 3 hrs to make sure that fly heads were submerged in fixative. The brains were dissected under stereomicroscope, fixed at room temperature for 30 min and incubated with 0.3% PBST (0.3% Triton X-100, 1×PBS) three times on a rotator, each time 20 min; then blocked for 1 hr at room temperature with blocking buffer (5% normal goat serum, 1×PBS, 0.1% Triton X-100), then incubated with anti-TH (1:200 dilute in blocking buffer) for 36 hrs at 4°C on a rotator. The primary antibody was removed and the brains were washed with 0.3% PBST three times on a rotator, each time 20 min, then incubated in goat anti-rabbit Alexa 488 (1:200 dilute in blocking buffer). Next, brains were washed with 0.3% PBST three times on a rotator,
each time 20 min, and finally mounted with FluorSave™ Reagent (Merck). Mounted brain samples were analyzed under a confocal microscope (Leica, TCS SP5).

4.2.7 Immunoblotting analysis of Drosophila samples

For Drosophila samples, immunoblotting analysis was carried out as previously described \cite{181} with minor adjustments. Twenty fly heads were homogenized in lysis buffer (50 mM Tris, 1%NP-40, 0.35% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, pH7.4) and lysed on ice for 20 min. The supernatants were collected after centrifugation (14,000 rpm, 10 min, 4°C) and denatured with 5× Laemmli sample buffer and then separated on 12% SDS-PAGE gels. The protein on the gels were then transferred to PVDF membranes (GE Healthcare, RPN303F) and processed for immunoblotting. Membranes were blocked with 5% nonfat milk and probed with primary and secondary antibodies. The bands were developed with an ECL kit (Pierce, 32106). The density of the bands was quantified by densitometry (ImageJ, NIH). For cell samples, lysis buffer was added and the cells were scraped off, transferred to microcentrifuge tubes and then lysed on ice for 30 min. The remaining steps were the same as those used for the fly samples.

4.2.8 Housing and husbandry of rats

The experiments performed on rats in the study were approved by the Hong Kong Baptist University Committee on the Use of Human & Animal Subjects in Teaching and Research. Inbred adult male Sprague-Dawley rats (220-260g) from the Laboratory Animal Service Centre, The Chinese University of Hong Kong were used. The rats were housed under standard conditions with 12 hrs light/dark cycles and constant room temperature of 22 ± 2°C and 60 ± 5%. They were fed with standard lab diet and
sterilized water ad libitum. The rats were accommodated to the environment for one week prior to further experiments.

4.2.9 Stereotaxic rotenone intoxication PD model and TGY treatment

Total 72 rats were used in the study, the rats were randomly allocated to 6 groups: Sham group (Infused with 1 µl DMSO). The rest of the rats were infused with rotenone and then assigned into the other 5 groups including Rotenone group (Model group, no further treatment); Sinemet group (Positive control group, received Sinemet equivalent to 42 mg/kg Levodopa); TGY low dosage group (TGY-L, received 1.5 g/kg TGY extract); TGY middle dosage group (TGY-M, received 3 g/kg TGY extract) and TGY high dosage group (TGY-H, received 6 g/kg TGY extract). The middle dosage of TGY is the equivalent to 7 folds of human dosage, the low dose is ½ of middle dose and the high dose is 2 folds of middle dose. All TGY groups received two weeks pretreatment prior to rotenone intoxication and two months post treatment.

The stereotaxic rotenone infusion was performed as previously described with minor modification [78]. Briefly, the rats were anesthetized with intraperitoneal injection of chloral hydrate (3.0% w/v in normal saline, 1 ml/100 g b.d.w.) and then placed and fastened on the stereotaxic frame (Stoelting, Wood Dale, US). Rotenone was dissolved in DMSO with a final concentration of 3 µg/µl, 1 µl of this solution was infused into the right ventral tegmental area (VTA) (AP: 5.3 mm; ML: 0.9 mm; DV: 8.0 mm) at a flow rate of 0.2 µl/min. The needle was retained for extra five minutes for the thorough diffusion of rotenone. After that, the needle was withdrawn slowly. Then rotenone was infused into the right SNc (AP: 5.3 mm; ML: 2.0 mm; DV: 8.0 mm) at a flow rate of 0.2 µl/min, with an additional five-minute needle retention. After operation, rats received
200 k Unit Penicillin by subcutaneous injection per day for total three days to prevent postsurgical infection.

4.2.10 Apomorphine (APO)-induced rotation

All groups of the rats were tested for APO-provoked rotations at the fourth and eighth week after drug administration. The rats were placed in an opaque plastic cylinder (diameter=30 cm) for 10 min acclimation, and then challenged with APO (3 mg/kg) via intraperitoneal injection. The following rotational behavior was then recorded for 35 min. The rotations were counted from 6 to 35 min. The rats were placed back into their cages after rotational testing.

4.2.11 Immunostaining of rat brain samples

Coronal sections (30 µm thickness) were cut throughout the striatum (from 1.0mm to 2.0mm caudal to the bregma) by using Shandon Cryotome SME Cryostat (Ramsey, Minnesota, USA). The sections were placed on the slides, air-dried, and then treated with 1% H$_2$O$_2$ for 10 min to quenched the endogenous peroxidase activity. After washed with 0.3% Triton-X100, the sections were blocked with diluted horse serum for 30 min, RT. After blocking, the sections were incubated with primary antibodies (TH antibody, 1:500 dilution; Iba-1, 1:500 dilution in 1% BSA/PBS) respectively, for 24 hrs at 4 °C.

For TH staining, the sections were incubated with ABC® Elite (VECTOR, Burlingame, USA) secondary biotinylated goat anti-rabbit IgG for 30 min at room temperature. Finally the sections were incubated with 3,3’-diaminobenzidine (DAB) for 5 min. The
sections were then air-dried and mounted with Leica mounting buffer. The slides were then observed under motic SMZ-171 microscope (Hong Kong, China).

For Iba-1 staining, the sections were incubated with Alexa Fluor® 594 goat anti-rabbit IgG (1:500 in 1% BSA/PBS) for 1hr at room temperature. The sections were then subsequently incubated with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min and finally mounted with VECTASHIELD hardset antifade mounting medium. The slides were then observed under Nikon 80i fluorescent microscope (Tokyo, Japan). All of the acquired pictures were processed and analyzed with ImageJ (NIH, USA).

4.2.12 Immunoblot analysis of rat brain samples
The ipsilateral striatum was dissected using a reported method[185] and homogenized with PBS containing 1mM PMSF (1mg brain added 10 µl PBS). The striatum was homogenized by sonication (120 w, 60 Hz, 2 s per cycle, total 25 blasts, BRANSON, USA) performed on ice. Then the lysate was centrifuged at the speed of 14,000 rpm for 30 min at 4°C. The supernatants were then collected and denatured at 95°C in sample buffer for at least 5 min. Denatured protein samples were then separated by 12% SDS-PAGE, transferred to PVDF membranes and incubated with the designated primary antibodies overnight at 4°C. After that, the blots were incubated with secondary antibodies at room temperature for 1 h. The protein signals were visualized by ECL kit (pierce, 32106) and quantified by densitometry analysis with ImageJ software.
4.2.13 Dopamine quantitation

For Drosophila, the brain dopamine levels were analyzed as reported [186]. After receiving designated treatment, 10 heads of flies from each group were homogenized in 100 µl pre-chilled 0.1M formic acid. The samples were centrifuged at 13,000 rpm, 4°C for 10 min. The supernatants were collected and freeze-dried, and then reconstituted with 100 µL ice-cold methanol; they were then centrifuged at 13,000 rpm, 4°C for 10 min. The supernatants were collected and subjected to LC/MS analysis.

For rat, the striatum dopamine level was determined with a reported method[187] with minor modification. Briefly, striatum with known weight was added with 10 times of 0.1 M HCl (10 µl/mg), homogenized with TissueLyser LT (QIAGEN, Hilden, Germany) for 1 min at 50 Hz. The samples were then centrifuged at 16,000 rpm for 10 min at 4 °C. Collected 1 ml supernatant for lysophalisation. The yields of the samples were redissolved by adding 100 µl 0.1 M HCl following 5 s vortexes and 5 s sonication on ice, and then 200 µl 0.1M HCl in MeOH were added, mixed by vigorous vortex for 20 s to precipitate protein. The samples were centrifuged at 16,000 rpm for 10 min at 4 °C. The supernatants were collected and injected into LC/MS. Calibration standards and QC samples were prepared by spiking serial diluted authentic compounds of each analyte to the blank brain sample. All procedures were carried out on ice or in cold room.

The LC/MS conditions were as follow: Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, a thermostatted column compartment, and an auto sampler was used to perform chromatographic analysis. Separations were achieved on a ZIC pHILIC 150×2.1mm, 5 µm column (Merck, New Jersey, USA) at 35 °C with mobile phase was consisted of ACN: ddH₂O
(50 : 50) supplemented with 10mM Ammonium acetate and 0.2% formic acid (A), and ACN: ddH₂O (95 : 5) supplemented with 10 mM Ammonium acetate and 0.2% formic acid (B) in a gradient elution washed. The gradient program was 0-1 min, 100% B; 1-15 min, 100-55% B; 15-16 min, 55-100% B; 16-25 min, 100% B.

An Agilent 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a jet stream electrospray ion source was used. The MS/MS system was operated under negative polarity mode and multiple-reaction-monitoring (MRM) mode. The MS conditions were set as follow: nebulizing gas (N₂) flow rate, 8 l/min; nebulizing gas temperature, 300 °C; sheath gas flow, 8 l/min; sheath gas temperature, 350 °C; nebulizer, 45 psi; capillary, 3500 V; Nozzle voltage, 1000 V. The MRM transitions of dopamine was m/z 154.0 → 91.2.

4.2.14 Cell culture and cell viability assay

Human neuroblastoma cells SH-SY5Y were maintained in DMEM (Invitrogen, 12800017), supplemented with 15% FBS (Invitrogen, 10099141). MTT assay was employed to evaluate cell viability as previously described[188]. SH-SY5Y cells were seeded in 96-well plates (5,000 cells/well). After 24 hrs, different treatments were performed. Then the media were removed and phenol red-free DMEM containing MTT (final concentration 0.5 mg/ml) was added, and cells were incubated for 4 hrs. The MTT solution was removed, and the cell crystals were dissolved using 100 µL of 20% sodium dodecyl sulfate (SDS) in 50% N, N-dimethylformamide (DMF). The intensity was measured using a plate reader at 570 nm with the reference of 620 nm.
4.2.15 Statistics analysis

Each experiment was repeated at least three times. The data was expressed as means ± SD. One-way ANOVA followed by Dunnett’s multiple comparison tests were conducted. All statistics analyses were performed in GraphPad Prism6 (GraphPad Software, Inc.).

4.3 Results

4.3.1 TGY antagonized rotenone toxicity in *Drosophila*

Rotenone has been reported as an environmental neurotoxin inducing PD-like phenotypes in *D. melanogaster*\(^{[88]}\). Based on the preliminary toxicity assay, male *w\(^{1118}\)* flies receiving 125 µM rotenone died within 14 days (Fig. 4-1 A) and the median lethal dose (LD\(_{50}\)) of rotenone was calculated to be 42 µM (Fig. 4-1 B). To test whether TGY is protective against rotenone intoxication, we fed the flies with rotenone and different dosages of TGY concurrently. As shown in Fig. 4-1 A, almost 80% of the flies died within 7 days and all died by the end of the experiment. However, the flies fed with TGY in addition to rotenone showed better survival rates. More than 80% of the flies treated with 100mg of TGY were alive on the 7\(^{th}\) day and no less than 30% of flies survived at the end of the experiment. In addition, TGY significantly alleviated motor dysfunction caused by rotenone intoxication (Fig. 4-1 C). In comparison with the normal flies, the flies intoxicated by the sublethal dosage of rotenone (42 µM) for 14 days exerted distinct motor function loss: most of the flies were unable to climb and stayed at the bottom of the vials. This phenotype was quantified by climbing assay. The result (Fig. 4-1 C) shows that, in the presence of rotenone, the flies commonly could not reach the finishing line within the time limit (50s). In contrast, in the absence of rotenone, only 15s were needed for the flies to reach the finishing line. The climbing
defect induced by rotenone was partially rescued by TGY treatment. The climbing ability of the flies treated with 100mg of TGY was significantly improved as indicated by the improvement in the climbing score (~20s).
**Fig. 4-1** TGY protected flies from rotenone intoxication. (A) Survival curves of the flies treated with rotenone with and without TGY were plotted. TGY increased the survival of the flies dose-dependently. (B) The LD50 of rotenone on w^{1118} was tested. (C) Climbing assay was performed to test the motor function of the flies. TGY alleviated the impairment of rotenone on motor function. Results were from at least three independent experiments except the LD50 assay. *p<0.05, **p<0.01, and ***p<0.001 compared with rotenone treated flies.

### 4.3.2 TGY reduced α-synuclein level and suppressed neurotoxicity in transgenic *Drosophila*

Abnormal α-synuclein accumulation is one of the major culprits causing PD. *Drosophila* overexpressing α-synuclein recapitulates the key features of human PD,[180, 181] such as loss of dopaminergic neurons, neuronal inclusion containing α-synuclein and locomotor defects.[81, 180, 181] These *Drosophila* PD models have been successfully utilized in testing the activities of small molecules and gene function.[189, 190] In the current study, we tested TGY on flies overexpressing α-synuclein. Wild type human α-synuclein (WT-α-synuclein) was pan-neurally overexpressed by elav-GAL4. The 30-day-old α-synuclein transgenic flies exhibited locomotor dysfunction as indicated by the climbing test scores. The aged α-synuclein transgenic flies took more than 30s to reach the finishing line while the normal control flies (elav-GAL4/+) spent less than 20s (Fig. 4-2 A). In addition to the behavioral results, the level of the marker protein of dopaminergic neurons, tyrosine hydroxylase (TH), was decreased strikingly in the presence of α-synuclein (Fig. 4-2 B). As a result of α-synuclein toxicity, the flies lost 40% of TH in comparison with normal control flies (Fig. 4-2 D). In contrast, TGY dose-dependently alleviated locomotor dysfunction (Fig. 4-2 A). The flies treated with 100mg of TGY spent less than 20s to reach the finishing line, which was similar to the normal controls. As shown in the typical blots, TGY prominently reduced the α-synuclein protein levels (Fig. 4-2 B and C) in the brains of the flies. Meanwhile, the
flies treated with TGY preserved more TH than untreated α-synuclein overexpressed flies (Fig 4-2 B and D). The results indicate that TGY alleviated the neurotoxicity triggered by α-synuclein overexpression and subsequently protected the dopaminergic neurons in the brains of the flies.

**Fig. 4-2** TGY alleviates the neurotoxicity induced by α-synuclein pan-neural overexpression. WT-α-synuclein was over expressed under the neuronal specific driver *elav-GAL4*. The flies were treated with TGY for 30 days. It was found that (A) TGY protects the flies from the impairment of motor function induced by WT-α-synuclein. (B-D) The level of WT-α-synuclein was decreased while the level of Tyrosine hydroxylase (TH), the marker protein of dopaminergic neuron, increased accordingly. Results were from at least three independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared with model flies without treatment.
4.3.3 TGY reduced α-synuclein induced dopaminergic neuron loss in transgenic *Drosophila*.

To further verify whether TGY could specifically counteract dopaminergic neuron degeneration caused by overexpression of α-synuclein, *Ddc-GAL4*, a dopaminergic neuron-specific *GAL4* driver, was employed to overexpress human WT-α-synuclein exclusively in the flies’ dopaminergic neurons. Full spectrum analyses of the dopaminergic neuron systems of the flies’ brains were performed. The flies were fed with TGY for 30 days and then decapitated. The heads were immunostained with TH through whole mount assay. TH-positive neurons in dorsomedial (DM) dopaminergic neuronal cluster of *Drosophila* brains were shown and counted (Fig. 4-3 A and B). The number of TH positive neurons suggested that TGY prevented dopaminergic neuron loss caused by WT-α-synuclein. The immunoblot data (Fig. 4-3 C and D) showed that TGY maintained the TH level in transgenic PD flies, which was consistent with the immunohistochemistry analysis data. To further confirm these findings, dopamine levels in the flies’ brains were determined using LC/MS analysis. The result (Fig. 4-3 E) showed that TGY preserved dopamine levels in the brains of transgenic *Drosophila*. The data of dopamine analysis and the TH protein level data obtained in immunoblot analyses together prove that TGY prevents dopaminergic neuron loss resulting from α-synuclein toxicity. In the climbing assay, TGY exhibited a protective effect in comparison with the untreated model flies (Fig. 4-3 F), which was also consistent with the previous findings.
Fig. 4-3 TGY rescued the loss of dopaminergic neurons caused by WT-α-synuclein over expression. WT-α-synuclein was over expressed by the dopaminergic neuron specific driver Ddc-GAL4. Flies were treated with TGY for 30 days. (A) Brains were dissected and immunostained with TH antibody to visualize the dopaminergic neurons. (B) The number of TH positive neurons in dorsal medial region was calculated (Five brains per group of each experiment). (C) The lysate of the brains were subjected to western blotting analysis and (D) the TH levels of each group were compared by densitometry analysis. To verify the histology and western blotting results, (E) the brains of the flies were extracted for LC/MS analysis to determine the dopamine levels. The results were consistent with (F) the data of climbing assay of each group of flies. Results were from at least three independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared with model flies without treatment.
4.3.4 TGY attenuated APO-provoked rotational behavior in rotenone-intoxicated PD rats

The neuroprotective effect of TGY was further examined in rotenone-intoxicated PD rats. Rats stereotaxically infused with rotenone to VTA and SNpc increasingly exerted rotational behavior that provoked by APO time dependently [78]. Four weeks after infused with rotenone, the rats exerted strong rotational behavior after challenged with APO (~10 circles/min, Fig 4-4 A). However, the rats receiving TGY significantly reduced the APO-provoked rotations. The APO-provoked rotations in rotenone-intoxicated rats receiving 3 and 6 g/kg TGY were reduced by nearly 60% compared with those rotenone-intoxicated rats without treatment. The rotational behavior was further tested at the end of treatment (8 weeks). As shown in Fig. 4-4 B, the rotenone intoxicated rats showed intensified APO-provoked rotations while TGY treatment notably prevented the deterioration.
Fig. 4-4 Effect of TGY on apomorphine (APO)-provoked rotational behavior. (A) TGY significantly prevented rotational behaviors provoked by APO in 4 weeks after rotenone intoxication, and (B) avoided exacerbation in 8 weeks post intoxication. n=5, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with the only rotenone intoxicated rats.

4.3.5 TGY alleviated dopaminergic neuron loss induced by rotenone intoxication.

To further verify whether TGY could prevent dopaminergic neuron loss, the ipsilateral part of the SNpc of each rats were harvested and subjected to immunoblotting analysis. The marker protein of dopaminergic neuron, tyrosine hydroxylase, was probed. The typical blot was shown in Fig. 4-5 A. As shown in the figure, rotenone infusion drastically reduced the level of TH, which indicated rotenone generated severe
impairment on the dopaminergic neurons. TGY alleviated the impairment dose dependently. As indicated by the densitometry analysis of the blots (Fig. 4-5 B) from five individual experiments, almost ~80% of the dopaminergic neurons were loss 8 weeks after rotenone intoxication, while the rats treated with 6 g/kg TGY preserved about 60% of the dopaminergic neurons. The results were further confirmed by histological examination of brain tissues. The typical TH DAB staining photos of SNpc and striatum from each group were shown in Fig. 4-6 and Fig. 4-7. The integrated intensity of TH immunostaining was examined by densitometry analysis. In SNpc, the results were quite consistent with the immunoblot result, the immunoreactivity of rotenone intoxicated rats showed ~80% loss TGY treated rats showed ~60% of immunoreactivity compared to that of the sham rats. In Striatum, ~50% loss of TH immunoreactivity of the rotenone intoxicated rats were recorded and TGY retained the TH immunoreactivity dose dependently. This result was further supported by the results of striatum dopamine levels (Fig. 4-8).
Fig. 4-5 TGY prevented decrease of TH in the SNc & VTA of the PD rats. Ipsilateral SNc and VTA of the rats were collected and detected (A) the TH protein by immunoblotting. (B) Densitometry analysis was performed. n=3, *p<0.05, **p<0.01, and ***p<0.001 compared with the only rotenone intoxicated rats.
Fig. 4-6 TGY alleviated the loss of TH immunoreactivity in the SNpc & VTA of the PD rats. The TH immunoreactivity was examined by DAB staining. Quantitative analysis of TH immunoreactivity in SNpc & VTA was performed through densitometry analysis. n=3, *p<0.05, **p<0.01, and ***p<0.001 compared with the only rotenone intoxicated rats. Scale bar: 1 mm
Fig. 4-7 TGY mitigated loss of TH immunoreactivity in the striatum of the PD rats. The TH immunoreactivity by DAB staining. Quantitative analysis of TH immunoreactivity in striatum was performed by densitometry analysis. n=3, *p<0.05, **p<0.01, and ***p<0.001 compared with the only rotenone intoxicated rats. Scale bar: 1 mm
Fig. 4-8 TGY diminished the depletion of dopamine in the striatum of the PD rats. The dopamine level of ipsilateral striatum was determined by LC/MS. n=3, *p<0.05, **p<0.01, compared with the only rotenone intoxicated rats.

4.3.6 TGY relieved neuroinflammation in rotenone-intoxicated PD rats

Rotenone infusion could result in neuroinflammation in rats [191-193]. Iba-1 immunofluorescent staining was carried out to evaluate the effects of TGY on the neuroinflammation induced by rotenone infusion. The brain sections of SNc and Striatum were stained with Iba-1 (red) and counterstain with DAPI (blue). The typical photos of SNc and dorsal striatum of each group were showed in Fig. 4-9 A and Fig. 4-10 A respectively. The results of Iba-1-positive cells counting revealed that rotenone intoxication activated microglia in SNc (Fig. 4-9 B) and striatum (Fig. 4-10 B) and TGY suppressed neuroinflammation induced by rotenone intoxication with the decrease of activated microglia.
**Fig. 4-9** TGY alleviated microglia activation in SNc. (A) The microglia marker protein Iba-1 in SNc was probed by immunostaining. (B) TGY significantly reduced the number of Iba-1-positive cells in SNc. n=3, *p<0.05, **p<0.01, and ***p<0.001 compared with the only rotenone intoxicated rats. Scale bar: 100 µm
Fig. 4-10 TGY reduced microglia activation in striatum. (A) Iba-1 in striatum was detected by immunostaining. (B) TGY apparently decreased the number of Iba-1-positive cells in dorsal striatum. \( n=3, \ast p<0.05, \ast\ast p<0.01, \) and \( \ast\ast\ast p<0.001 \) compared with the only rotenone intoxicated rats. Scale bar: 100 µm
4.3.7 TGY reduced rotenone-induced apoptotic cell death in SH-SY5Y cells

To further investigate the neuroprotective mechanisms of TGY, we tested the anti-apoptosis effect of TGY on human dopaminergic SH-SY5Y cells by adding rotenone to induce apoptotic cell death [194]. SH-SY5Y cells were treated with 20 μM of rotenone and co-treated with different dosages of TGY for 24 hrs. The cell viability was tested by MTT assay. Result of the MTT assay indicates that TGY prevented cell death induced by rotenone (Fig. 4-11 A). In immunoblot analysis, the pro-survival protein Bcl-2 and the apoptosis marker, cleaved-caspase3 were detected. TGY preserved the level of Bcl-2 and reduced the cleavage of caspase3, which indicates TGY alleviated rotenone-induced apoptosis (Fig. 4-11 C-E).

Fig. 4-11 TGY prevented apoptosis induced by rotenone on SH-SY5Y cells. It was found that (A) Rotenone induced cell death was counteracted by TGY. (B) TGY extract is safe to the SH-SY5Y cells ≤100 μg/ml. (C) Apoptotic cell death, as indicated by unregulated cleaved Caspase-3 and down regulated pro-survival protein Bcl-2, caused by rotenone treatment, was alleviated by TGY. (D, E) The Bcl-2 and cleaved-Caspase 3 levels in different groups were compared by densitometry analysis. Results were obtained from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with rotenone-intoxicated cells without treatment.
4.4 Discussion and conclusion

In this part of the study the anti-PD activity of TGY was evaluated in \textit{in vivo} and \textit{in vitro} models of PD. Treatment with TGY significantly suppressed rotenone-induced death and locomotor dysfunction of \textit{Drosophila}. Feeding with TGY notably reduced α-synuclein protein levels and comparatively prevented loss of dopaminergic neurons in the brains of \textit{Drosophila}. In stereotaxic rotenone intoxication rats, TGY significantly prevented dopaminergic neurons loss, alleviated neuroinflammation, and improved the behavior. Meanwhile, the anti-apoptosis activity of TGY was also verified in SH-SY5Y cells. These results provide experimental evidences supporting the application of TGY for the treatment of PD.

TGY is traditionally used in the form of decoction In the TGY water extract, flavonoids and iridoids, such as Baicalin and Geniposide, are the most abundant small molecules. These compounds have been shown to have anti-inflammatory, anti-oxidative and anti-apoptotic activities [195-198]. All of the compounds in TGY constitute the material basis of its pharmacological activity. How the components of TGY concert anti-PD activity deserves further investigation.

In this study, the insecticide rotenone was introduced to established \textit{in vivo} and \textit{in vitro} PD models. Rotenone was reported as an inhibitor of mitochondrial complex I and an inducer of dopaminergic lesion [199]. Through treating \textit{Drosophila} with lethal and sublethal doses of rotenone, a PD model was established to evaluate the anti-PD activity of TGY (\textbf{Fig. 4-1}). When using this model, we found that special attention must be paid to the variability in susceptivity to rotenone of different \textit{Drosophila} strains. In the present study, we performed the experiments all with the same \textit{w}\textsuperscript{1118} strain [200]. The
stereotaxic rotenone intoxication rat PD model was successfully employed to evaluate the neuroprotective effect of TGY in prevention of dopaminergic neuron loss. The anti-neuroinflammation activity of TGY was also examined by this rat PD model. Furthermore, we treated SH-SY5Y cells \[^{201}\] with rotenone to induce apoptotic cell death. Mild anti-apoptotic cell death activity of TGY was observed (Fig. 4-11), indicating anti-apoptotic mechanism plays an important role in the neuroprotective activity of TGY.

In PD, \(\alpha\)-synuclein is the major pathogenic protein. The mutations in this protein accelerate its aggregation then contribute to the onset and progress of PD \[^{36}\]. Thus, reducing the formation and increasing the clearance is a reasonable strategy for developing a therapy for PD. *Drosophila* is a widely used model organism in studying neurodegenerative diseases \[^{202}\]. By using the GAL4/UAS system \[^{203}\], ectopic overexpression of human \(\alpha\)-synuclein in the brain of *Drosophila* was established as a genetic PD model \[^{189}\]. In the current study, we utilized elav-GAL4 \[^{204}\] to drive pan-neural expression of \(\alpha\)-synuclein. Overexpressed \(\alpha\)-synuclein impaired dopaminergic neurons as indicated by decreased TH level in immunoblot analysis and defective ability in the climbing assay. TGY treatment efficiently reduced \(\alpha\)-synuclein and rescued the dopaminergic neurons (Fig. 4-2). However, all of the neurons, including dopaminergic neurons, were affected by \(\alpha\)-synuclein overexpressed by elav-GAL4. Hence we used a dopaminergic neuron-specific GAL4 driver, Ddc-GAL4, to exclusively express \(\alpha\)-synuclein in the dopaminergic neurons of adult flies \[^{205}\]. We implemented a broad spectrum of analyses focusing on dopaminergic neurons. The results demonstrate that TGY protected dopaminergic neurons from \(\alpha\)-synuclein impairment (Fig. 4-3). From these two *Drosophila* PD models, we provided sufficient evidence that TGY
decreases α-synuclein in the neurons and thereby, prevents dopaminergic neuron loss consequently.

In conclusion, our data indicate that TGY is neuroprotective in PD models. This corroborates its ongoing use in traditional Chinese medicine to treat PD-like symptoms. Evidence that it works immediately initiates a flood of further inquiries as to how it works. For example, how does TGY impact the misfolded and oligomerized α-synuclein that is thought to be more toxic than the monomer \[^{206}\]? How does TGY regulate abnormalities in lysosome or proteasome function that is important in clearance of α-synuclein \[^{207}\]?
CHAPTER 5. Geniposide, a principal component of TGY, promotes autophagy both in vivo and in vitro

5.1 Introduction

Macroautophagy, herein referred to as “autophagy”, is an intracellular degradation system that degrade and recycle unwanted cellular components and long-lived proteins in the cooperation with lysosome to maintain cellular homeostasis [208]. It is a conserved cellular mechanism from yeast to plants and animals that has an pivotal role in various aspects including cellular remodeling, adaptation to environment alteration and even determination of life span [209]. In the pathogenesis of disease, autophagy plays an adaptive role to protect organisms against various pathologies such as infections, heart diseases, cancer, aging and neurodegeneration [210]. Given its critical role in pathophysiology, autophagy is becoming a main target for drug discovery and development [211].

In neural system, a general consensus has been achieved that autophagy is cytoprotective against neuronal cell death that triggered by pathological and physiological stress [212-216]. Genetic analysis related to autophagy provides evidences to its importance. Loss of autophagy genes such as Atg6 and Atg7 resulted in diminished motor function, nonetheless, Atg knockout mice has a short life span [217]. In handling the progressive accumulation of aberrant proteins, autophagy cooperated with proteasomal degradation system to clear unwanted proteins in neuronal cells since autophagy is adept at targets larger aggregated proteins and proteasomal degradation system is efficient in eliminating short-lived proteins [218]. Physiologically, neurons have extremely large expanses of dendritic and axonal cytoplasm. As post-mitotic cells,
neurons are highly dependent on the global lysosomal systems in handling dysfunctional organelles and cellular waste from accumulating without the aid of cell division. In other words, neurons are particularly rely on the proteolytic clearance in maintaining cellular homeostasis \cite{219}. Ubiquitinated protein and neuron degeneration could take place in absence of competent autophagy \cite{217,220}.

Abnormal accumulation of proteins is being increasingly recognized playing detrimental role in the pathogenesis of various neurodegenerative disorders including Alzheimer’s disease, Huntington’s disease and Parkinson’s disease \cite{221}. The ubiquitin-proteasome (UPS) and autophagy-lysosome pathway (ALP) are the two key degradation systems in handling the abnormal accumulation of unwanted proteins \cite{222}. Since ALP is able to degrade accumulated proteins, autophagy turns into a potential therapeutic target for neurodegenerative disorders. Discovering pharmacologic inducers of autophagy in phytochemicals is feasible to develop neuroprotective therapeutics for the treatment of neurodegenerative disorders \cite{223}.

Geniposide, an iridoid, is a main phytochemical in *Gardeniae Fructus* (栀子), which is one of the 11 herbs of TGY formula. Geniposide was reported to be protective in AD \cite{224}, has anti-inflammatory activity \cite{225} and anti-oxidative activity \cite{167} In the previous part of the study, we found that TGY promoted the clearance of α-synuclein and Geniposide is one of the five most abundant quantified small phytochemicals. Geniposide could be the key neuroprotective component of TGY. In this part of the study, the activity in enhancing autophagy of Geniposide was evaluated.
5.2 Materials and methods

5.2.1 Reagents and antibodies
Chloroquine (C6628), anti-FLAG® M2 (F1804) antibody, and doxycycline (D9891) were purchased from Sigma-Aldrich. Rapamycin (R5000) was purchased from LC Laboratories. ACTB/β-actin (sc-47778) was purchased from Santa Cruz Biotechnology. Anti-LC3 (2775), anti-SQSTM1/p62 (5114), and mouse anti-rabbit IgG were purchased from Cell Signaling Technology. α-Syn (SNCA) antibody (610786) was purchased from BD Transduction Laboratories. BECN1 (beclin 1, NB110-87318) and HMGB1 (NBP1-40650) antibodies were purchased from Novus Biologicals. LDH Cytotoxicity Detection Kit was purchased from Roche Applied Science. DMEM (11965-126), DMEM/F12 (12634-010), Neural basal medium (21103-049) horse serum (16050-122), fetal bovine serum (FBS) (16000-044), G418 (10131-035), goat anti-mouse-HRP (626520), goat anti-rabbit-HRP (G21234) were purchased from Invitrogen.

5.2.2 Cell lines and cell culture
N2a cells were cultured in DMEM, supplemented with 10% FBS. PC12 cells were grown in DMEM, supplemented with 10% FBS and 5% horse serum. SH-SY5Y cells were maintained in DMEM/F12, supplemented with 15% FBS. N2a cells constitutively expressing GFP-LC3 were cultured in DMEM, supplemented with 10% FBS and 200µg/ml G418.

5.2.3 Protein samples preparation and immunoblot analysis
After designated treatments, the cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.35% sodium deoxycholate, 1 mM EDTA, 1% NP40, 1 mM
PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin). The sample were lysed on ice with occasional vortex for 30 min, and then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant were collected and denatured at 95 °C in sample buffer for 5 min. The denatured samples were then separated on 15% SDS-PAGE gels and transferred to PVDF membrane. Membranes were then blocked with 5% non-fat milk and probed with appropriate primary and secondary antibodies. The protein signals were finally visualized with ECL kit and the protein bands density were quantified by ImageJ program.

5.2.4 Fluorescence analysis of cell puncta

Cells were fixed with 4% paraformaldehyde (Sigma, 158127) for 10 min, and then mounted with VECTASHIELD hardset antifade mounting medium. The mounted samples were then subjected to confocal microscopy analysis. Numbers of GFP puncta were counted as previously described [226]. Briefly, the GFP puncta in the cells were counted manually and at least 30 cells of each treatment were randomly chosen for counting. The presented typical data were selected from one of the three independent repeated experiments.

5.2.5 Primary neuron culture

The E16 pregnant rat was anesthetized by intraperitoneal injection with 3% chloral hydrate in normal saline (1ml/100g bdw). The uterus containing embryos were dissected and transferred to a Petri dish, and then the embryos were collected on a new petri dish containing ice cold PBS. The brains of the embryos were then dissected under stereoscope and the cortices of the brains were placed in a Petri dish containing ice cold
10% FBS DMEM. The meninges of the cortices were removed carefully and then transferred to a 15 ml tube. Total 5 ml of digestion solution (1.25% Trypsin diluted with DMEM/F12 medium) were added into the tube containing meninges-free cortices and then incubated at 37°C for 15 min. The digestion was stopped by adding 10% FBS DMEM/F12 supplemented with 10 µg/ml DNase (Sigma, DN25). The solution of tissue mixture were then pipetted up and down thoroughly to dissociate the cells until no obvious tissue blocks were observed and the tissue mixture can pass the tip of the pipet easily. After dissociation, the tissue mixture was passed through a 70 µm filter (BD Falcon, USA) into a 50 ml tube. The filtrate was centrifuged at 800 rpm at room temperature for 3 min, the supernatant was decanted and then the cells were re-suspended with Neurobasal medium (Invitrogen, 21103049) supplemented with B27 supplement (Gibco, 17504), 25 µM L-Glutamic acid (sigma, G1251) and 1% PSN (15640-055). For cell counting, total 10µl of the re-suspended cells were diluted 10 times with typan blue. The viable cells without staining with typan blue were then counted under microscope. The cells were seeded on poly-D-lysine (Sigma, P0988) pretreated culture plates. For imaging, the cells were seeded in a low density (1×10^5 cells/well of 24-well plate). For biochemistry analysis, the cells were seeded in a high density (3×10^6 cells/well of 6-well plate). Twelve hours later, medium were replaced completely by Neurobasal medium supplemented with B27, 2 mM L-Glutamine (Gibco, 25030) and 1% PSN. Three days later, 5 µM Ara-C (Sigma, C6645) was added. The medium was half changed 24 hrs later. Cultures were fed every 3 days by half-replacement of the old medium with fresh medium. Cultures were maintained for at least seven days for neuron differentiation and maturation.
5.2.6  *Drosophila* strains and culture

*Cg-GAL4* that drives transgene expression in *Drosophila* fat body and *UAS-GFP-mCherry-Atg8a* were obtained from Bloomington *Drosophila* Stock Centre. Flies were raised at 25°C on standard cornmeal medium, under 50%~70% relative humidity with 12 hrs dark-light cycle. *Cg-GAL4* flies were crossed with *UAS-GFP-mCherry-Atg8a* to express *GFP-mCherry-Atg8a* in *Drosophila* fat body and crossed with *w*¹¹¹⁸ to obtain control flies.

5.2.7  Confocal analysis of Atg8a-mCherry-EGFP puncta in *Drosophila* fat body

Crossed flies were allowed to lay eggs for 1 hr, 90 hrs later, the L3 larvae were collected for further treatment. Geniposide and Cory B were firstly dissolved in DMSO and then diluted to the desired concentration with ddH₂O and then mixed thoroughly with instant fly food. The L3 larvae were placed in the prepared food and received treatment for 6 hrs. After treatment, the fat body lobes of the larvae were harvested with fine forceps and fixed with 4% PFA in PBS at room temperature for 15 min. Fat body lobes were then washed with PBS three times and subsequently mounted with VECTASHIELD hardset antifade mounting medium. The slides were then subjected to confocal microscopy analysis. Z stack serial scanning were performed, the images of GFP and mCherry were both recorded and merged. Only the red puncta, which indicated the mature autolysosomes, were counted.

5.2.8  Statistics analysis

The data was expressed as means ± SD. One-way ANOVA followed by Dunnett’s multiple comparison tests were used. All statistics analyses were performed in GraphPad Prism6 (GraphPad Software, Inc.).
5.3 Results

5.3.1 Geniposide upregulated LC3II level in neuronal cell lines

The five most abundant small phytochemicals were screened to identify autophagy inducers. Among these five phytochemicals, Wogonoside has already been reported as an autophagy inducer \[227\]. Geniposide was finally identified as an autophagy inducer. It was firstly discovered that Geniposide increased LC3II, a marker protein of autophagy, in neuroblastoma N2a cells dose dependently (Fig. 5-1 A and B). Other two neuronal cell lines were applied to confirm this phenotype. The results showed that Geniposide increased LC3II level in the other two neuronal cell lines, namely PC12 and SH-SY5Y cells (Fig. 5-1 C-F). The toxicity of Geniposide on neuronal cells was evaluated by MTT assay on N2a cells and the result revealed that Geniposide is safe up to 200 \(\mu\)M. These results indicated that Geniposide could be a potent neuronal autophagy inducer without affecting cell viability.
Fig. 5-1 Geniposide upregulated LC3II level in different neuronal cell lines. The cells were treated with different concentrations of Geniposide for 24 hours and cell lysates were subjected to immunoblotting analysis. The levels of LC3II were probed and analyzed by densitometry analysis. The following cell lines were used, (A and B) mice neuroblastoma N2a cells, (C and D) rat pheochromocytoma PC12 cells, and (E and F) human neuroblastoma SH-SY5Y cells. The cytotoxicity of Geniposide was evaluated by N2a cells through MTT assay. (G) Cells receiving up to 200 µM Geniposide treatment for 24 hours were viable. n=3, *p<0.05, **p<0.01
5.3.2 Geniposide increased LC3II level in primary rat cortical neurons

To further verify the results obtained on neuronal cell lines, Geniposide was further tested on primary cultured rat cortical neurons. The primary cortical neurons were obtained from E16 embryonic SD rats. The primary neurons were fed by neurobasal medium supplemented with B27 for at least 7 days for maturation prior to further treatment. On DIV 7, the purity of the primary neurons was verified by staining the cultured cells with neuronal marker MAP2 and microglia marker Iba-1 (Fig. 5-2). The result proved that the primary neurons were of ideal purity for further experiments. On DIV 10, the primary neurons were treated with various concentrations of Geniposide for 24 hrs. The proteins of LC3II and SQSTM1 were probed via immunoblotting analysis. Results revealed that Geniposide increased LC3II level, similar to that in neuronal cell lines, in primary cultured cortical neurons. Moreover, concurrent decrease of SQSTM1 protein level was also observed. (Fig. 5-3 A and B)
Fig. 5-2 Confirmation of the primary cortical neurons’ purity by MAP2 and Iba-1 staining. E16 SD embryonic rat cortical neurons were cultured in neurobasal medium for 7 days prior to staining. Cells were fixed in 4% PFA and stained with neuronal marker MAP2 (Green) and microglia marker Iba-1 (Red) and counterstained with DAPI, and then observed under fluorescent microscope. Photos were recorded under 20× magnification.
Fig. 5-3 Geniposide increased LC3II level in primary cultured neurons dose dependently. At DIV10, the rat embryonic primary cortical neurons were treated with various concentrations of Geniposide for 24 hours and the cell lysate were subjected to immunoblotting analysis. (A) The protein of SQSTM1 and LC3II were probed and (B) quantitatively analyzed by densitometry analysis. Results showed that Geniposide increased LC3II level while decreased SQSTM1 level of the primary neurons in a dose dependent style. n=3, *p<0.05, **p<0.01.
5.3.3 Geniposide promoted autophagic flux in N2a cell.

Blocking autophagic flux such as inhibiting the fusion of autophagosome and lysosome could also manifest an increase of LC3II \(^{[228]}\). In other words, compounds that inhibit the turnover of autophagy could also increase LC3II. Therefore, Geniposide was co-treated with and without, a lysosomal inhibitor, chloroquine (CQ) to measure the amount of LC3-II delivered to lysosomes. Furthermore, an autophagosome formation inhibitor, 3-methyladenine (3MA) was also employed to confirm the ability of Geniposide in inducing autophagy. N2a cells stably expressing GFP-LC3 were treated with Geniposide (25 µM) with and without CQ (20 µM) and 3MA (5 mM) for 24 hrs. The cells were then fixed with 4% PFA and the images were recorded under confocal microscope (Fig. 5-4 A). The GFP-LC3 puncta of the cells receiving different treatments were counted as described in section 5.2.4. As shown in the data (Fig. 5-4 A and B), cotreatment of Geniposide and CQ drastically increased the number of GFP-LC3 puncta comparing to the cells treated by CQ only. This result indicated that Geniposide is an autophagic flux inducer but not an autophagosome lysosome fusion inhibitor. The autophagy inducing activity of Geniposide was abolished by autophagosome formation inhibitor 3MA, which further verified Geniposide is an autophagic flux inducer. N2a cells were treated with the same experiment setup and the lysates were analyzed by immunoblotting. The results (Fig. 5-4 C and D) were consistent with fluorescent analysis.
Fig. 5-4 Geniposide promoted autophagic flux without affecting formation of autolysosome. (A) N2a cells stably expressing GFP-LC3 were treated with 25 µM Geniposide with or without 20 µM CQ and 5 mM 3MA for 24 hrs. Cells were fixed with 4% PFA and photos were recorded by confocal microscope. (B) GFP-LC3 puncta of at least 30 cells were counted. The numbers of the puncta were from one representative experiment from three repeated experiments. Same experiments were performed on N2a cells and cell lysates were subjected to immunoblotting analysis. (C) Typical blots were shown and (D) densitometry analysis was performed. The results indicated that Geniposide upregulated LC3II level without blocking autolysosome formation and can be blocked by 3MA, an autophagy inhibitor. (Compared with Ctrl: *p<0.05, **p<0.01, ***p<0.001; Compared with CQ: #p<0.05, ##p<0.01, ###p<0.001. Results were obtained from three independent experiments.)
5.3.4 Geniposide enhanced autophagic flux in *Drosophila*

The in vitro study suggested that Geniposide is a potent autophagy inducer. To further test whether Geniposide could induce autophagy in vivo, *Drosophila* carrying Atg8α-mCherry-EGFP transgene [229] and *Cg-GAL4* that drives specific expression of transgene in fat body [230] were used in the study. Flies were crossed and allowed to lay eggs. After 90 hrs post oviposition, the L3 larvae were treated with 100 μM Geniposide for 6 hrs to induce autophagy. Corynoxine B was use as positive control. The red puncta, which indicate matured autolysosomes, were counted. As shown in the results (Fig. 5-5), The larva fed with Geniposide generated more matured autolysosome compared with the larva fed with blank food as indicated by the red puncta. This result proved that Geniposide could induce autophagic flux *in vivo.*
Fig. 5-5 Geniposide enhanced autophagic flux in *Drosophila* fat body. *Drosophila* carrying Atg8a-mCherry-EGFP transgene were crossed with *Cg-GAL4* flies. After 90 hours of oviposition. The L3 larvae were transferred to the food vial containing 100 μM Geniposide or Corynoxine B (positive control) and treated for 6 hrs. The fat bodies were harvested and fixed with 4% PFA, and then subjected to confocal microscope analysis. The typical images were shown and the numbers of mature autolysosome (red puncta) were counted as described in section 5.2.7 and compared with ctrl. The results proved that Geniposide promoted the maturation of autolysosome in *Drosophila* fat body. n=3, *p<0.05,**p<0.01
5.3.5 Geniposide reduced α-synuclein level and suppressed neurotoxicity in transgenic *Drosophila*

The neuroprotective effects of Geniposide were tested on *Drosophila* overexpressing α-synuclein in neurons. Flies were treated with Geniposide for 30 days and the heads were harvested then lysed and subjected to immunoblotting analysis. Geniposide decreased the α-synuclein level dose dependently and alleviated the concomitant loss of dopaminergic neurons as indicated by TH level (Fig. 5-6 A and B). The motor function of the transgenic flies was evaluated at the end of treatment. The flies treated with Geniposide showed improved climbing activity comparing with the flies without treatment (Fig. 5-6 C).
Fig. 5-6 Geniposide reduced α-synuclein level and prevented loss of dopaminergic neurons in Drosophila PD model. The flies pan-neurally overexpressed with α-synuclein were treated with different concentration of Geniposide for 30 days. The heads were harvest and the lysate were subjected to immunoblotting analysis. (A) TH and α-synuclein were probed and (B) quantitative analyzed. (C) The motor function of the flies was evaluated by climbing assay at the end of the treatment. The results showed that TGY reduced α-synuclein level, prevented the reduction of TH and
alleviated motor function defect simultaneously. n=3, *p<0.05, ***p<0.001, ****p<0.0001, #P<0.05.

5.3.6 Geniposide induced autophagy in neuronal cells in an mTOR-independent manner

Autophagy process is executed rigorously in eukaryotes from yeast to mammalian cells. The regulative signaling pathways were reported extensively [231-235]. In order to understand the molecular mechanism of Geniposide in autophagy induction, the impact of Geniposide on the mTOR pathway, a canonical autophagy signaling pathway, was examined. N2a cells were treated with various concentrations of Geniposide for 6 hrs. Rapamycin (Rapa), a potent autophagy inducer that acting as mTOR inhibitor was used as positive control. The results showed that both Geniposide and Rapamycin induced autophagy as indicated by the increased LC3II and mature Cathepsin D (CatD) levels. However, Geniposide treatment did not alter the phosphorylation of mTOR or influence P70s6K, which is a substrate of mTOR. In contrast, Rapamycin dramatically inhibited p-mTOR and p-p70s6k. These results indicated that Geniposide is not inducing autophagy via mTOR inhibition.
Fig. 5-6 Geniposide induced autophagy without inhibiting mTOR. N2a cells were treated with different concentrations of Geniposide, Rapamycin was used as positive control. The results showed that Geniposide induced autophagy dose dependently as indicated by the LC3II level and mature CatD. This process did not inhibit mTOR activity as indicated by normal p-mTOR level and the normal phosphorylation of its substrate p70s6K.

5.4 Conclusion and discussion

In this part of the study, Geniposide was proved an autophagy inducer both in vitro (Fig. 5-1 and 5-3) and in vivo (Fig. 5-4). It was also confirmed that Geniposide is not an
mTOR inhibitor and it induce autophagy through mTOR independent pathway (Fig. 5-7). The neuroprotective effect of Geniposide was evaluated on Drosophila PD model. Geniposide was proved able to reduce α-synuclein, the pathogenic protein involved in PD, and alleviated the concomitant dopaminergic neuron loss (Fig. 5-6).

Mounting evidences prove that defects in autophagy contribute to the pathology of numerous neurodegenerative disorders including but not limited to Alzheimer’s disease and Parkinson’s disease [236]. Targeting up-regulation of autophagy could be a therapeutic strategy against neurodegenerative disorders caused by inadequate clearance of unwanted protein and organelles [237]. Searching for pharmacologic agents from small molecules that activate autophagy is a feasible strategy to develop therapeutics for neurodegenerative disorders including PD [223]. A complete autophagic flux could be divided into 4 steps, vesicle nucleation, phagophore elongation, docking and fusion of autophagosome and lysosome, and maturation of autolysosome & degradation of contents [238]. Small molecules could modulate autophagy through targeting relative signaling pathways pertaining these different steps. The autophagy signaling pathways have been studied intensively and reported that inhibitors of mTOR [239, 240] and class I PI3K [241, 242], inhibitor of inositol monophosphatase [243, 244], activators of AMPK [245, 246] and Ca\(^{2+}\) channel antagonists [247, 248] could activate autophagy. The current results of the study manifested that Geniposide induce autophagy in mTOR independent manner (Fig. 5-7). The molecular target of Geniposide in autophagy induction needs further investigation.

Last but not least, Geniposide was proved that could induce autophagy (Fig. 5-4) and reduce α-synuclein in Drosophila (Fig. 5-6). However, further evidences are needed to
prove that Geniposide could reduce α-synuclein via activate autophagy. In conclusion, Geniposide is identified an autophagy inducer and is proved to be neuroprotective on *Drosophila* PD model.
CHAPTER 6. Pharmacokinetics interactions between TGY and Sinemet

6.1 Introduction

Traditional herbal medicines are quite popular, especially in Chinese population around the world [249-251]. The safety issues of concurrent administration of herbal medicines and Western medicines deserve more attention. Albeit the pharmacokinetics and pharmacodynamics of Western medicines are well characterized, most of the herbal medicines are lacking such information because of the complexity on their components since most of the herbal medicines are constituted with several herbs. Concomitant use of herbs has been reported to mimic, magnify, or oppose the effect of drugs [252]. This is supported by documented reports of herb-drug interactions. For example, Glycyrrhizin from Glycyrrhiza glabra decreases plasma clearance, increases AUC [253], and increased plasma concentration of prednisolone [254]. Xiao Chaihu Tang decreases AUC of prednisolone [255]. Jiawei Xiaoyao San extends the elimination half-life of 5-Fluorouracil and increases its volume of distribution in blood [256]. Scutellariae radix alleviates mefenamic acid-induced stomach adverse effect and potentiates the anti-inflammatory effects [257].

In PD treatment, medicaments containing Levodopa, such as Sinemet (Carbidopa-Levodopa) are the first-line drugs used to increase dopamine concentrations in the brain in the symptomatic treatment of PD more than 40 years (www.michaeljfox.org). Dopamine replacement with Levodopa, compared with other available dopaminergic therapies showed the greatest improvement in motor function. Moreover, responsiveness to Levodopa is a diagnostic criterion for PD. In clinical practice,
Levodopa slows the progression of disability and reduces mortality. Importantly, Levodopa is one of the well-tolerated drugs to treat PD, particularly among the elderly \[^{258}\]. However, most patients are prone to manifest complications within the first a few years from the initiation of therapy, and develop both motor symptoms and non-motor symptoms \[^{259}\].

Most of the patients adopting TCM therapy are already taking conventional drugs such as Sinemet (Carbidopa-Levodopa), and then continue to take both types of drugs at the same time \[^{260-262}\]. This is not unusual. It has been reported that nearly two-thirds of PD patients worldwide resort to various kinds of complementary or alternative medicine, including TCM, which may possibly influence symptoms of PD, and/or the effectiveness of dopaminergic therapy \[^{263}\]. It was reported that Baicalein, one of the key compound from TGY, exerts neuroprotective effect against Levodopa induced neuronal cell death \[^{264}\]. However, no study has provided evidence about the safety and efficacy of taking Levodopa medicaments and TCM simultaneously, even though this is a matter of considerable interest.

In this part of the study, the pharmacokinetics interaction between TGY and Sinemet was studied in order to delineate the pharmacokinetic profiles of Levodopa and the selected main components of TGY after co-administration of TGY and Sinemet.
6.2 Materials and methods

6.2.1 Materials and reagents
Acetonitrile (MS grade) was purchased from RCI Lab scan Ltd. (Bangkok, Thailand), analytical grade formic acid was purchased from Sigma. HPLC grade methanol and ethanol were obtained from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore MilliQ-Plus system (Millipore, Bedford, MA, USA). All other reagents were of at least analytical grade.

Reference compounds of Baicalin and Geniposide with purity over 98% were purchased from Chengdu Preferred Biotechnology Co. Ltd. (Chengdu, China). Levodopa with purity above 98% was purchased from Sigma (1361009). 4’,5,7-trihydroxyflavanone was kindly provided by Prof. ZUO Zhong from the Chinese University of Hong Kong. The purities were confirmed by UPLC-DAD analysis based on peak area normalization.

6.2.2 Preparation of standards solutions and quality control (QC) samples
Master stock solutions of Baicalin and Geniposide were dissolved in MeOH/ddH₂O (1:1, v/v) respectively to reach 0.2 mg/ml. Levodopa was dissolved in 0.1M HCl in MeOH with a concentration of 0.1 mg/ml. The stock solutions were stored in -80°C.

For validation assay, a stock mixture containing 0.1 mg/ml Baicalin and Geniposide were prepared by mixing the master stocks of each compound. The stock mixture was then further diluted with MeOH to reach final concentration of 5 µg/ml for both of Baicalin and Geniposide. Levodopa was diluted with 0.1M HCl in MeOH to the final concentration of 5 µg/ml. The internal standard (IS) was prepared by dissolving 4’, 5, 7-trihydroxyflavanone in MeOH at a final concentration of 5 µg/ml.
Calibration standards and QC samples were prepared by spiking working standards to drug-free rat plasma. Serial diluted mix-standards were prepared, 20 µl of each concentration of the standards was spiked in 100 µl plasma, and then 10 µl IS was added. Levodopa calibration standards were prepared with the same procedure. QC samples were prepared at 100, 300 and 600 ng/ml for Baicalin, Geniposide and Levodopa. The spiked plasma samples of calibration standards and QC was then processed following the sample preparation method described in section 6.3.3.

6.2.3 Sample preparation

Plasma samples were obtained from pharmacokinetic study, 10 µl of IS working standard and 200 µl MeOH were added (180 µl for calibration standards and QC samples). The sample was vortex vigorously for 20 s and then subjected to sonication (60 Hz, 5 s). After sonication, the samples were centrifuged at 16,000 rpm for 15 min at 22°C, the supernatant was collected in glass sample vial and then injected into the LC/MS/MS system for analysis.

6.2.4 Analytical method

Chromatographic analysis was performed on an Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, a thermostatted column compartment, and an auto sampler. Separations were achieved on an ACQUITY UPLC BEH C18 column (2.1 mm × 100 nm, 1.7 µm, Waters, Milford, USA) at 40 °C with mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) in a gradient elution washed. For Baicalin and Geniposide, a gradient mobile phase program was used as follow: 0-6 min, 15-45% B; 6-6.1 min, 45-100% B; 6.1-10
min, 100% B; 10-10.1 min, 100-15% B; 10.1-14 min, 15% B; The flow rate was 0.35 ml/min. For Levodopa, the gradient program was: 0-1.8 min, 2% B; 1.8-2 min, 2-100% B; 2-5 min, 100% B; 5-5.1 min, 100-2% B; 5.1-8 min, 2% B. The flow rate was 0.3 ml/min.

An Agilent 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a jet stream electrospray (ESI) ion source was used. The MS/MS system was operated under negative polarity mode and multiple-reaction-monitoring (MRM) mode. The MS conditions were as follow: nebulizing gas (N2) flow rate, 8 l/min; nebulizing gas temperature, 300 °C; sheath gas flow, 9 l/min; sheath gas temperature, 350 °C; nebulizer, 45 psi; capillary, 3500 V; Nozzle voltage, 1000 V. The MRM transitions and other MS parameters of each analyte were optimized by direct injection of the authentic standards into the MS spectrometer respectively. The data acquisition was controlled by MassHunterB.03 software (Agilent Technologies).

**6.2.5 Method validation**

The method validations of the developed methods were validated following the guidance of Bioanalytical Method Validation from the U.S. Food and drug Administration (FDA) [265].

**Specificity:** The specificity of the method was assessed by comparison of the chromatograms of blank solvent sample, blank rat plasma samples, standards in plasma samples, standards in solvent samples and the rat plasma samples after receiving designated treatments.
**Calibration curve linearity and range:** Calibration standards samples for generation of calibration curves were prepared as described in section 6.3.2. Serial diluted standards and IS were spiked in plasma and processed as described in section 6.3.3. The calibration curves were constructed by plotting the IS normalized peak area versus concentration for each analyte. The acceptable linearity was defined as the regression coefficient ($R^2$) was higher than 0.95. The lower limit of quantification (LLOQ) was used as the lowest standard on the calibration curve. The LLOQ was of an accuracy ± 20% of the nominated concentration, a precision that ≤ 20% of the coefficient variation (CV) and with a signal-to-noise response ratio ≥ 5:1. The lower limit of detection (LLOD) was presented as the lowest concentration that has a signal-to-noise ratio ≥ 3:1.

**Accuracy, precision, and recovery:** Intra-day and inter-day accuracy and precision were determined. Three concentrations QC samples and five replicates of each concentration were analyzed within one day and three days. Accuracy ≤ 15% of the nominal concentration and precision ≤ 15% CV were accepted. Peak area of each analyte spiked prior and post extraction was used to calculate the extraction recovery rate.

**Stability:** The stability of sample in auto-sampler (5°C) was determined by comparing the peak area of sample from two runs with a 24 hrs interval.

**6.2.6 Pharmacokinetics interaction of TGY and Sinemet in rats**

The pharmacokinetics study was approved by the Hong Kong Baptist University Committee on the Use of Human & Animal Subjects in Teaching and Research. Inbred adult male Sprague-Dawley rats (260 – 300 g) from the Laboratory Animal Service Centre, The Chinese University of Hong Kong were used. High dosage of TGY extract
(6 g/kg, the high dosage used in rotenone-intoxicated rats, which is equivalent to 188.04 mg/kg Baicalin and 78.90 mg/kg Geniposide) and Sinemet (Calculated by Levodopa: 10 folds of the human dosage ‘250mg/kg’, which is equivalent to 42 mg/kg Levodopa) used in pharmacodynamics study was adopted in the pharmacokinetics study. All of the tested drugs were prepared as suspension in ddH₂O.

The plasma pharmacokinetic time profiles of the analytes were determined. Rats (n=4 for each treatment) were orally dosed with TGY, TGY + Sinemet and Sinemet respectively. Bloods (300 µl/timepoint) were sampled from the retro-orbital plexus at 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 hr and collected with heparinized centrifuge tubes. The blood samples were then centrifuged at 16,000×g for 5 min at room temperature. The plasma samples (100 µl/sample) were collected and stored in -80 °C before further process. The concentration versus time pharmacokinetic profile of each analyte was analyzed by PKSolver. Non-compartmental model was used in calculating the pharmacokinetic parameters. The area under the curve from zero to infinity (AUC₀₋∞), the elimination half-life (t₁/₂), and total systemic clearance (CL_total) were calculated. The maximum concentration (C_max) and the time attaining C_max (T_max) were referred to the experimental data.

6.3 Results

6.3.1 Method validation

Specificity: The representative multiple reaction monitoring (MRM) chromatograms of blank rat plasma (Fig. 6-1), blank rat plasma spiked with analytes (Fig. 6-2) and rat plasma sample obtained after oral administration of TGY and Sinemet respectively (Fig 6-3) were obtained. As shown in Fig. 6-1, no interfering peaks were observed at the
retention times of the analytes, which indicates a measure up selectivity of the assay. In the Baicalin MRM chromatogram of plasma sample, extra peaks were recorded (Fig. 6-3 C). The retention time of these peaks are well separate and the quantitation of Baicalin was not affected. Calibration curve linearity and range: The calibration curves of the three analytes were achieved with at least six different concentrations of each analyte. The data of linearity, linear ranges with the LLOQs and LLODs were listed in Table 6-1. All calibration curves of the analytes obtained good linearity within the ranges as indicated by $R^2 > 0.99$. Accuracy, precision, and recovery: The accuracy and precision results were shown in Table 6-1. Both intra-day and inter-day experiments were performed following the guideline [265]. Rate of recovery of the analytes were also tested and listed in Table 6-1. Stability: The stability of the analytes of QC samples was evaluated and the data were shown in Table 6-1. The results indicated that Geniposide and Baicalin were stable in plasma samples in 5 °C autosampler for 24 hrs. Levodopa is quite unstable and easy to be oxidized and sensitive to the alteration of temperature. Thus all samples containing Levodopa were processed on ice and subjected to LC/MS analysis as quick as possible.
Fig. 6-1 Representative MRM chromatograms of blank rat plasma. MRM transitions of the (A) Levodopa; (B) Geniposide and (C) Baicalin were examined. (D) The blank plasma sample was also examined. No interfering peaks were detected at the retention time of the three analytes.
Fig. 6-2 Representative MRM chromatograms of blank rat plasma spiked with analytes at different concentrations. (A) Levodopa 3.66 µM; (B) Geniposide 4.47 µM; and (C) Baicalin 2.92 µM. (D) 4’,5,7-trihydroxylflavone was used as internal control.
Fig. 6-3 Representative MRM chromatograms of rat plasma samples obtained after oral administration of Sinemet or TGY. (A) Levodopa; (B) Geniposide; (C) Baicalin and (D) 4’,5,7-trihydroxyflavanone were detected in the sample.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Range (µM)</th>
<th>Equation</th>
<th>R²</th>
<th>LLOD (µM)</th>
<th>LLOQ (µM)</th>
<th>Precision (RSD, %, n=5)</th>
<th>Spike recovery (RSD, %, n=3)</th>
<th>Stability (RSD, %, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geniposide</td>
<td>0.02-6.83</td>
<td>Y=9.0098X-391.83</td>
<td>0.9945</td>
<td>0.01</td>
<td>0.02</td>
<td>7.34</td>
<td>7.74</td>
<td>3.85</td>
</tr>
<tr>
<td>Baicalin</td>
<td>0.02-5.82</td>
<td>Y=49.897X-2113</td>
<td>0.9977</td>
<td>0.01</td>
<td>0.02</td>
<td>7.86</td>
<td>5.79</td>
<td>2.62</td>
</tr>
<tr>
<td>Levodopa</td>
<td>0.14-8.46</td>
<td>Y=6.2762X+41.049</td>
<td>0.9979</td>
<td>0.07</td>
<td>0.14</td>
<td>11.32</td>
<td>5.21</td>
<td>3.97</td>
</tr>
</tbody>
</table>
6.3.2 Pharmacokinetics interaction between TGY and Sinemet

The established LC/MS/MS method was applied to study the pharmacokinetics interaction between TGY and Sinemet following the protocol described in section 6.2.6. After administration of TGY and Sinemet under the designated dosages by oral gavage concurrently and respectively, the plasma samples were prepared and analyzed by LC/MS/MS. The respective plasma concentration versus time profiles of Levodopa, Baicalin and Geniposide were plotted (Fig. 6-4) and the pharmacokinetics parameters were calculated through non-compartmental analysis (Table 6-2). Notably, concurrent administration of TGY dramatically affected the pharmacokinetics profile of Levodopa (Fig. 6-4 A). As reflected by the pharmacokinetics parameters (Table 6-2), the $C_{\text{max}}$ and $\text{AUC}_{0-\infty}$ were both reduced greater than 70% while the total systemic clearance ($\text{CL}_{\text{total}}$) was three folds increased. Meanwhile, the pharmacokinetic profiles and parameters of Baicalin and Geniposide were also monitored. Concomitant administration of Sinemet as well altered the pharmacokinetics disposition of Geniposide. Albeit no overt alteration of pharmacokinetics profile was observed (Fig. 6-4 C) The $C_{\text{max}}$ and $\text{AUC}_{0-\infty}$ of Geniposide were diminished while the $\text{CL}_{\text{total}}$ was augmented significantly (Table 6-2)
Table 6-2 Plasma pharmacokinetic parameters of Levodopa, Baicalin and Geniposide.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>Pharmacokinetic parameters (single oral dose, n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$T_{\text{max}}$ (h) $C_{\text{max}}$ (µM) AUC$<em>{0-\infty}$ (µM h) $t</em>{1/2}$ (h) $\text{Cl}_{\text{total}}$ (L/h/kg)</td>
</tr>
<tr>
<td>Levodopa</td>
<td>Sinemet</td>
<td>1.38 ± 0.75  10.71 ± 2.17  41.56 ± 3.51  1.26 ± 0.43  1.02 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>TGY+Sinemet</td>
<td>1.15 ± 1.90  2.34 ± 1.09<strong>b  11.52 ± 1.77</strong>*b  3.93 ± 1.96  3.72 ± 0.63**a</td>
</tr>
<tr>
<td>Baicalin</td>
<td>TGY</td>
<td>9.00 ± 1.15  14.89 ± 3.56  253.30 ± 86.76  10.41 ± 4.97  0.81 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>TGY+Sinemet</td>
<td>9.50 ± 1.00  15.17 ± 2.91  443.80 ± 218.5  17.66 ± 10.17  0.51 ± 0.26</td>
</tr>
<tr>
<td>Geniposide</td>
<td>TGY</td>
<td>0.38 ± 0.14  36.98 ± 14.90 133.89 ± 35.14  5.92 ± 0.74  0.62 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>TGY+Sinemet</td>
<td>0.69 ± 0.38  16.01 ± 11.55 84.13 ± 20.36  5.85 ± 1.27  0.98 ± 0.22**a</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$ time to reach maximum plasma concentration; $C_{\text{max}}$: maximum plasma concentration; AUC$_{0-\infty}$: area under the curve from 0 to infinity; $\text{Cl}_{\text{total}}$: total systemic clearance.

* Significant increase compared to TGY or Sinemet treatment alone.
** Significant decrease compared to TGY or Sinemet treatment alone.

$p<0.05$, $**p<0.01$, and $***p<0.001$. Data were presented in mean ± SD, Student’s $t$-test with Welch corrections were performed.
Fig. 6-4 Plasma concentration vs. time profiles of the analytes. TGY and Sinemet were orally administrated concurrently and respectively. The plasma concentration vs. time profiles of (A) Levodopa; (B) Baicalin and (C) Geniposide were plotted. Data were presented as mean ± SD, n=4.
6.4 Conclusion and discussion

The pharmacokinetics interaction between TGY and Sinemet were studied. Evidently, co-administration of TGY affected the pharmacokinetics of Levodopa, which is the main composition of Sinemet. (Fig. 6-4 A). The pharmacokinetics characters of Geniposide, a marker phytochemical of TGY, were also affected (Table 6-2). These evidences, therefore, suggest that in clinical application, Levodopa containing medicaments such as Sinemet should avoid been taken concomitantly with TGY. The luminal intestinal absorption of Levodopa was executed by the neutral and dibasic amino acids exchanger [267]. This transporter could be the primary target by which TGY altered the absorption of Levodopa. The underlying mechanisms of herb-drug interactions are complicated. Induction and/or inhibition of metabolic enzymes is one of the major mechanisms that responsible for herb-drug interaction [268]. Through induction of CYP family, herbs could lower the plasma concentration of some conventional drugs [269]. Herbs that affecting hepatic enzymes could also cause pharmacodynamics effects [270-273]. Inhibition and/or induction of the ATP-binding cassette (ABC) family of drug transporters and efflux proteins is another major mechanism account for herb-drug interaction. The ABC family of drug transporters is important in drug absorption and elimination [274-276]. Modulation of the activity of these drug transporter or competitive affinity as substrates could result in alteration in pharmacokinetic profile of the drug [277-280]. Alteration of gastrointestinal (GI) and renal functions could also result in herb-drug interactions. Such as changes in GI pH or other factors may alter the absorption of some drugs [281-284]. Affecting the renal function could result in change of renal elimination [285, 286]. Taken together, the possibilities lead to the result could be some of the phytochemicals in TGY extract 1) directly interacted with Levodopa in the digestive system; 2) compete the transporter of Levodopa or 3) upregulate the activity of DOPA.
decarboxylase activity, 4) antagonize the inhibition activity of Carbidopa in the circulation system. Further experiments are needed to elucidate the underlying mechanism. Besides the pharmacokinetics herb-drug interaction, pharmacodynamics interaction is also important. Herb-drug interaction could take place through synergy, addition or antagonism between herb and drug [257, 287-290]. The current study provides evidences on the pharmacokinetics interaction of TGY and Sinemet. The underlying mechanism and their pharmacodynamics interaction deserve further investigation.
Chapter 7. General discussion and conclusion

7.1 Material basis of TCM formula

Following TCM theory, physicians adopt the formula principles of chief, deputy, assistant and envoy herbs and then select the appropriate dosage of herbs based on the causes and symptoms of diseases [291,292]. The chemical constituents of TCM formula play an important role in constructing the therapeutic material basis of pharmacodynamics [293]. The efficacy of TCM formula is the result of each composition herb. Some interactions among different chemicals from herbs of TCM formula in the process of decocting could result in significant alterations in the constituents of the decoction [294-296]. This could be the essence of the compatibility of herbs and exacerbate the complexity on constituents of decoction. Therefore, incisive analysis on the chemical constituents of TCM formula is vital in understanding the material basis of TCM formula and developing proper quality control method for TCM formula [297-299]. Advancement in analytical methodology, especially in chromatography and mass spectrometry, provides us powerful tools to study the chemical constituents of TCM formula [300,301].

In our study, an UHPLC/Q-TOF-MS and HPLC-ELSD method was developed and applied to qualitatively and quantitatively determine the complex phytochemicals of TGY. Total 28 phytochemicals were identified, of which 20 were quantified. Saccharides were also identified and quantified. However, nearly 50% of the TGY extract remains unquantified. One of the possible reasons is that most of the commercialized reference compounds are isolated from single herbs, while the decocting process may result in alterations in the constituents of the decoction. The
result of the study provides a better understanding of the material basis of TGY. Further efforts are needed to achieve full components recognition of TGY.

7.2 TGY in PD treatment

Tianma Gouteng Yin (TGY, 天麻鉤藤飲), a famous decoction of traditional Chinese medicine (TCM), is first recorded in the medical book, New Concepts for the Diagnosis and Treatment of Miscellaneous Illnesses(《中醫雜病證治新義》), compiled by the TCM physician HU Guangci [90]. This formula belongs to the category that dispel wind, which is now utilized to calm liver Wind, clear Heat and promote blood flow in terms of TCM theory [91]. In its clinical application, TGY is originally applied to cure hypertension that related to agitation of liver wind according to TCM theory [90, 302-304]. Nowadays, TGY is frequently used to treat Parkinsonian-like symptoms because in TCM theory, agitation of liver wind is also a principal pathogenesis factor of PD. Interestingly, in some cases of PD, hypertension is also one of the main non-motor symptoms [305-307]. These evidences bridge the Western medicine and Chinese medicine on the knowledge of PD. However, just as most of the TCM formula, the underlying pharmacology of TGY is still lacking experimental evidences.

In our study, the anti-PD activity of TGY was verified both in vivo and in vitro. In Drosophila PD models, TGY mitigated rotenone induced toxicity and promoted α-synuclein clearance. In stereotaxic rotenone intoxication rats, TGY exerted neuroprotective effects in terms of preventing dopaminergic neurons loss and alleviating neuroinflammation. TGY alleviated rotenone induced apoptosis in SH-SY5Y cells.
Based on the evidence that TGY promoted the clearance of α-synuclein, the phytochemicals of TGY were screened for autophagy inducer. Defects in autophagy contribute to the neurodegeneration of PD \[308\]. Autophagy inducers could alleviate neurodegeneration via promoting autophagy \[215, 309\]. The results of the study verify that Geniposide is an autophagy inducer both \textit{in vivo} and \textit{in vitro} and is neuroprotective in transgenic \textit{Drosophila} PD model. Collectively, these experimental evidences prove that TGY is neuroprotective in PD models and corroborate its application for the treatment of PD.

7.3 Herb-drug interaction

Herb-drug interaction could result in detrimental adverse effects. Herb-drug interactions discussed here is not about chemical interaction between a drug and a phytochemical from an herb that generating toxic substance. But rather the pharmacokinetics interaction that an herb derived phytochemical cause either an increase or decrease in the amount of drug in the circulatory system. In such herb-drug interaction, a decrease in the amount of drug could happen by herb derived phytochemicals binding with the drug and then interrupting its absorption, or by stimulating the enzymes that degrade the drug and prepare its elimination. Moreover, an increase in the drug concentration could take place due to the phytochemicals facilitate the absorption of the drug, or inhibit its metabolization. Decrease in drug concentration by virtue of an interaction could diminish the efficacy of the drug while increase in drug concentration could make it passively overdose.

In Chinese population, TCM is quite popular, the potential for herb-drug interaction increases. In China, TCM herbal formulas have higher risk to interact with Western
drugs because of the popularity of integrated TCM and Western medicine therapies. In our study, herb-drug interactions between the two marker phytochemicals, Baicalin and Geniposide, and one of the most popular drugs used in PD treatment, Sinemet, were studied. The pharmacokinetics data showed that co-administration of TGY could affect the pharmacokinetics of Levodopa, the main component of Sinemet, for the first time. This information suggest that in clinical practice, TGY should avoid being administrated with Levodopa containing medicaments at the same time.

7.4 Limitation of the study
The results of the study provide a comprehensive understanding on TGY’s pharmacological characteristics for PD treatment. However, there are some limitations of the current study: 1) 50% of TGY extract, by weight, remains unquantified; 2) the underlying mechanism that Geniposide promotes autophagy remains unclear; 3) the mechanism by which TGY suppressed the absorption of Levodopa is not clear; 4) the pharmacodynamic interaction between TGY and Sinemet has not yet been studied; 5) long-term toxicity of TGY Sinemet co-administration is not yet studied. It is expected that, in future, the resolvement of these unanswered questions, a more comprehensive understanding on the pharmacological activity of TGY in PD treatment could be achieved.

7.5 Conclusion
Conclusively, the chemical compositions of TGY decoction were first identified and quantified by a developed analytical method; The efficacy of TGY in PD treatment was evaluated on various PD models and indicated that TGY is neuroprotective; Furthermore, The underlying mechanism on neuroprotection of TGY was investigated by assessing the autophagy inducing activity of Geniposide, which is a core
phytochemical of TGY; The pharmacokinetics interaction between TGY and Sinemet, a commonly prescribed drug in PD treatment, was tested and it was discovered that the absorption of Levodopa, main component of Sinemet, could be suppressed by TGY.
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Publication List

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Manuscripts Submitted to Referred Journals (2015)


Patents:


Abstracts Presented at International Conferences:


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