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Evaluation of neuroprotective effect of *Clitoria ternatea* on biomarkers of experimentally induced Parkinsonism in mice

Biochemistry

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ABSTRACT

In the present study catecholaminergic neurotoxin MPTP was used to lesion dopaminergic pathways in the experimental animal model of Parkinson's disease (PD). The main objective of the study was to evaluate the effect of Clitorea ternatea (CT) on biomarkeres of Parkinson's disease in mice. Swiss albino mice (adult male) were divided into control group, MPTP-treated group (30 mg/kg body weight, i.p in 0.9% saline, single dose), CTE treated groups (200 and 400 mg/kg body weight) after MPTP exposure for 21 days. Striatal total antioxidant capacity (TAC), serum and striatal xanthine oxidase (XO) activity, the marker of oxidative stress, and the DNA repair enzyme PARP (poly(ADP-ribose)polymerase) were estimated. There was a significant increase ($p<0.05$) in XO and PARP activities and a significant decrease ($p<0.05$) in TAC with MPTP treatment. CT treatment significantly ($p<0.05$) reduced these changes showing a significant neuronal protection. MPTP-challenged mice treated with 200 and 400 mg/kg of CT indicated significant neuronal protection by decreasing the XO and PARP activities. MPTP challenged mice treated with CT restored the TAC level significantly ($p<0.05$). Comparatively CT treatment at higher dose (400 mg/kg) was more effective in neuronal protection as indicated by biochemical parameters. In conclusion, CT extract enriched with bioflavonoids showed a significant neuroprotective effect against MPTP-induced neurotoxicity in mice by decreasing XO and PARP activities in and increasing TAC activity.

KEYWORDS:

Parkinson's disease, *Clitoria ternatea*, Neuroprotective effect

INTRODUCTION:

Parkinson's disease (PD) is an incurable progressive neurodegenerative disorder of predominantly idiopathic origin which occurs due to death of dopaminergic neurons in the substantia nigra pars compacta (SNc), a region of the brain that controls motor activity by projecting dopaminergic axons to the striatum [1]. Tremor, muscle rigidity, bradykinesia (slowness in initiating and carrying out voluntary movement), postural and gait abnormalities are early symptoms of PD [2]. More advanced stage of PD is associated with cognitive and behavioral problems, including dementia, although it is not generally fatal. Studies have shown that PD affected 1,000,000 Americans, 1% of individuals older than 60 years [3]. Genetically, younger age groups (< 21) are also affected by PD.

The classical pathological findings associated with PD are the presence of Lewy bodies (microscopic protein deposits of alpha-synuclein) in the substantia nigra region, and accompanied by loss of nerve cells in the ventral tier [4]. Selective depletion of dopaminergic inputs to the striatum was found to be the most important neurochemical characteristic of PD [5]. Dopamine deficiency is the hallmark feature in PD. It is one of three major neurotransmitters known as catecholamines, which helps the body to respond to stress and for fight-or-flight response. Loss of dopamine negatively affects the nerves and muscles controlling movement and coordination, resulting in the major symptoms characteristic of Parkinson's disease. Dopamine also appears to be important for efficient information processing, and deficiencies may also be responsible for problems in memory and concentration that occur in many patients [6].

There are many anti-parkinsonism drugs released in the stores, but these currently available drugs offer temporary relief for the symptoms of Parkinsonism's disorder, but unable to arrest or reverse the neuronal degradation caused by the disease, some even causes undesirable adverse effects like anorexia, nausea, and vomiting, [2]. Currently the interest of natural products enriched with bioflavonoids including *Clitoria ternatea* L. as a treatment of PD has been growing [7]. The benefits of flavonoids are generally thought to be derived from their antioxidant and free radical–scavenging properties [8]. *Clitoria ternatea* (CT) has beneficial effect on multiple disease states, including cancer, cardiovascular disease, and neurodegenerative disorders [9, 10, 11]. It is interesting to know that CT has many pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, antidiabetic, insecticidal, and platelet aggregation-inhibiting [12], and even it has been used as a traditional medicine in India to enhance memory and intelligence [13, 14]. So it will be interesting to estimate the effect of *Clitoria ternatea* (CT) on Parkinson's disease.

MATERIALS AND METHODS

Clitoria ternatea extract

Fresh whole parts of *Clitoria ternatea* were collected, cleaned, dried in shade, and were powdered. Then the powder soaked in 80% ethanol (1:4) in rotatory shaker at room temperature, the extracted solution was filtered through Whatman No.1 filter paper and dried using rotator evaporator at 40°C, and finally the crude extract was obtained and stored in 4°C for further use.

Animals

Swiss albino mice (adult male) weighing 25-30 g were obtained from USM, Penang, Malaysia. The animals were housed under standard laboratory conditions (25±2°C; 12 hrs of light and dark cycles), and were given access to food, water and libitum, and acclimatized for seven days before starting the experiment. All the animal handling procedures for the experiment have been approved by AIMST university human and animal ethics committee (AUHAEC 78/FOM/2012).

Animals were randomly assigned into four groups of 8 mice in each group. Control group (Group 1) received normal saline alone orally, MPTP group (Group 2) received MPTP (Toronto Research Chemicals inc., Canada) dissolved in 0.9% saline 30 mg/kg body weight intraperitoneally (i.p) single dose, MPTP + CTE (200mg/kg) group (Group 3), received MPTP (30mg/kg body weight i.p.) and CTE 200 mg/kg body weight / day orally, and MPTP + CTE (400mg/kg) group

(Group 4), received MPTP (30mg/kg body weight i.p.) and CTE 400 mg/kg body weight/day orally. The plant extract treatment was done for a period of 21 days. On completion of the experimental period the animals were sacrificed by decapitation under sodium pentobarbital anesthesia (40 mg/kg body weight, i.p injection). Brain tissues were excised immediately, the striatum region was, isolated according to (Glowinsky and Iverson, 1966) method, and immersed in ice cold saline, then homogenized using PBS for Parkinsonism biochemical studies. Blood samples were collected in EDTA tubes by cardiac puncture, centrifuged at 20,000 rpm for 20 min, and the supernatants were collected and stored at -20°C. Brain striatal tissue samples were homogenized in PBS (pH 7.4), using a teflon homogenizer, and the homogenates were stored at -20°C for further use for biochemical studies. From the homogenate samples, poly (ADP-ribose) polymerase (PARP), xanthine oxidase (XO), and total antioxidant capacity (TAC) were assayed. While from the serum sample PARP and XO were assayed. Where (Trevigen, Inc. USA) assay kit, (abnova) assay kit, and (OxiSelect, Cell Biolabs, Inc.) assay kit were used for PARP, XO and TAC assays respectively.

Statistical analysis

SPSS statistical software was used for statistical analysis. Paired sample T-Test analysis was performed to compare between the control, MPTP, and the CT treated groups. One way ANOVA test was used to indicate the significant differences between different doses tested. The mean ± sd values were calculated for each group. Results were statistically analyzed based on three replicates done for each of the three biochemical studies.

RESULTS

PARP assay

Results of PARP activity of brain striatal tissue indicated a significant ($p<0.05$) increase in PARP levels after exposure to MPTP. The mean value of PARP enzyme level in the control group was found to be 0.0803±0.003 U/mg protein, while it was found to be 0.2126±0.005 U/mg protein in MPTP group. Treatment with CT (200 mg/kg) significantly ($p<0.05$) reduced these changes and the PARP enzyme level was decreased and the mean value was found to be 0.1301±0.005 U/mg protein. Treatment with CT (400 mg/kg) significantly ($p<0.05$) reduced these changes and the enzyme level was decreased, and the mean value was found to be 0.1023±0.002 U/mg protein (Table 1).

There was a significant ($p<0.05$) increase in serum PARP levels after exposure to MPTP, the mean value of PARP enzyme level in the control group was found to be 1.539±0.07 U, and 4.341±0.184 U for the MPTP group. Treatment with CT (200 mg/kg) significantly ($p<0.05$) reduced these changes and the PARP level was decreased and the mean value was found to be 2.800±0.105 U. Treatment with CT (400 mg/kg) significantly ($p<0.05$) reduced these changes and the enzyme level was decreased, and the mean value was found to be 1.841±0.098 U (Table 1).

When comparison was done between the two doses of CT treatment, a significant difference ($p<0.05$) in PARP activity in brain striatum and serum was noticed and the 400 mg/kg was found to be more effective.

Table 1. Effect of CT on PARP activity in brain striatal tissue and serum.

Treatment	PARP activity	
	Brain striatal tissue (U/mg of protein)	Serum (U)
Control (group 1)	0.0803±0.003	1.539±0.07
MPTP (group 2)	0.2126±0.005*	4.341±0.184*
MPTP + CTE (200 mg/kg) (group 3)	0.1301±0.005*	2.800±0.105*
MPTP + CTE (400 mg/kg) (group 4)	0.1023±0.002*	1.841±0.098*

* Values are significantly different ($p<0.05$) from control.

The PARP activity was determined following universal colorimetric PARP assay method, The values are expressed in mean ± s d (n=6).

XO assay

Results of brain striatal tissue indicated that there was a significant ($p<0.05$) increase in XO levels after exposure to MPTP. The mean value of XO level in the control group was found to be 0.0026±0.0003 mU/ml, and 0.0041±0.0001 mU/ml for the MPTP group. Treatment with CT (200 mg/kg) significantly ($p<0.05$) reduced these changes and the XO level was decreased, the mean value was found to be 0.0031±0.0001 mU/ml. Treatment with CT (400 mg/kg) significantly ($p<0.05$) reduced these changes and the level was decreased, and the mean value was found to be 0.0029±0.0005 mU/ml (Table 2).

There was a significant ($p<0.05$) increase in serum XO levels after exposure to MPTP, the mean value of XO level in the control group was found to be 0.0024±0.0001 mU/ml, while it was found to be 0.0073±0.0003 mU/ml in MPTP group. Treatment with CT (200 mg/kg) significantly ($p<0.05$) reduced these changes and the XO level was decreased and the mean value was found to be 0.0066±0.0004 mU/ml. Treatment with CT (400 mg/kg) significantly ($p<0.05$) reduced these changes and the enzyme level was decreased, and the mean value was found to be 0.0035±0.0002 mU/ml (Table 2).

When comparison was done between the two doses of CT treatment, a significant difference ($p<0.05$) in XO level in brain striatum and serum was noticed and the 400 mg/kg was found to be more effective.

Table 2. Effect of CT on XO activity in brain striatal tissue and serum.

Treatment	XO activity	
	Brain striatal tissue (mU/ml)	Serum (mU/ml)
Control (group 1)	0.0026±0.0003	0.0024±0.0001
MPTP (group 2)	0.0041±0.0001*	0.0073±0.0003*
MPTP+CTE(200mg/kg)(group 3)	0.0031±0.0001*	0.0066±0.0004*
MPTP+CTE(400mg/kg)(group 4)	0.0029±0.0005*	0.0035±0.0002*

* Values are significantly different ($p<0.05$) from control.

The XO level was determined following XO assay Abnova method, and the values are expressed in mean ± s d (n=6).

TAC assay

There was a significant ($p<0.05$) decrease in TAC levels in brain striatal tissues after exposure to MPTP, the mean value for the control group was 0.232±0.008 mM, while the mean value for MPTP group was 0.144±0.007 mM in tissue. Treatment with CT (200 mg/kg) significantly ($p<0.05$) reduced these changes and the TAC was increased, the mean value was found to be 0.203±0.006 mM in the tissue. Treatment with CT (400mg/kg) significantly reduced these changes and the TAC was increased ($p<0.05$), and the mean value for MPTP + CTE (400 mg/kg) was found to be 0.218±0.006 mM in the tissue (Table 3).

When comparison was done, there was no significant difference ($p>0.05$) in the TAC activity between in CT (200 mg/kg) treatment and CT (400 mg/kg) treatment.

Table 3. Effect of CT on TAC in brain striatal tissue.

Treatment	TAC (mM)
Control (group 1)	0.232±0.008
MPTP (group 2)	0.144±0.007*
MPTP + CTE (200 mg/kg) (group 3)	0.203±0.006*
MPTP + CTE (400 mg/kg) (group 4)	0.218±0.006*

* Values are significantly different ($p<0.05$) from control.

The TAC was determined following TAC assay kit (oxiselect™, cell biolabs. Values are expressed in mean ± s d (n=6).

DISCUSSION

Exogenous administration of MPTP was found to induce neuronal damage, which closely resembles Parkinson's disease [15]. In the present study, MPTP (30 mg/kg b.wt, i.p single dose) was used to induce parkinsonism in adult male mice [16]. Animals were divided into 4 groups of 8 mice in each group. Control group (Group 1), MPTP group (Group 2), MPTP + CTE (200 mg/kg) group (Group 3), MPTP (30 mg/kg b.wt. i.p.) and CTE 200 mg/kg body weight/day orally, and MPTP + CTE (400 mg/kg) group (Group 4) received MPTP (30 mg/kg b.wt. i.p.) and CTE 400 mg/kg body weight/day orally. The plant extract treatment was done for a period of 21 days.

The results of PARP assay indicated that serum and brain striatal PARP significantly increased ($p<0.05$) in the MPTP-challenged animals, but treatment with CT significantly decreased ($p<0.05$) the PARP levels, indicating the protective effect and it was more effective in higher dose of CT.

PARP is activated by binding to DNA ends or strand breaks, and its activity is strictly proportional to the number of DNA breaks, whereas it is totally inactive in the absence of DNA breaks [17, 18, 19]. PARP activation depletes NAD via poly(ADP-ribosyl)ation of nuclear proteins, and ATP is further depleted in an effort to resynthesize NAD, leading to cell death by energy depletion. Consistent with this notion is the observation that replacement of cellular energy stores provides protection against MPTP neurotoxicity [20, 21]. Although energy depletion is thought to play a prominent role in PARP-mediated cell death [17, 18]. PARP is activated primarily by single-strand nicks of DNA that typically occur after free radical damage, but it is insensitive to double-strand DNA ends that typically occur during apoptosis [22]. The role of PARP activation in other forms of cell death, such as cerebral ischemia, glutamate excitotoxicity, cytokine, and free radical-mediated damage to pancreatic islet cells as well as cardiac damage after occlusion of coronary arteries, suggests that PARP may be a critical choke point in a variety of important pathologic conditions [17, 18].

A significant increase ($p<0.05$) in the levels of xanthine oxidase (XO) was observed in the serum and brain striatum of MPTP treated groups after 21 days of MPTP exposure. This is an indication of excessive oxidative stress and resultant dopaminergic neuronal damage. Treatment with CTE significantly reduced ($p<0.05$) these MPTP-induced changes and enzyme levels in both serum and striatum of CTE treated groups (200 and 400 mg/kg body weight). During oxidative stress in the neuronal cells there is an increase in intracellular Ca^{2+} levels in the brain [23]. This increased intracellular Ca^{2+} can induce the irreversible conversion of xanthine dehydrogenase (XDH) to XO, which in turn catalyzes the oxidation of xanthine to provide a source of O_2^- In addition, auto-oxidation of dopamine in brain could also serve as a source of superoxide anion [24]. These mechanisms could be the main reasons for the increased level of XO and reduction in activity of SOD leading to an overload of oxygen radicals and repression of antioxidant enzymes with MPTP exposure.

The output of this study indicated that the MPTP administration significantly lowered the TAC in the striatum of mice as compared to control mice. Total antioxidant capacity (TAC) depleted mice have shown more vulnerability of MPTP insult. We found that the intraperitoneal administration of MPTP significantly decreased ($p<0.05$) TAC in the striatum. Concurrent treatment with CTE (both doses 200 and 400 mg/kg body weight) significantly increased ($p<0.05$) TAC in striatum. The increased oxidative stress is considered a cardinal feature of MPTP neurotoxicity. It has been documented that, decreased SOD and CAT enzymatic activities were observed in MPTP treated animals [25]. SOD protects tissues by catalyzing the removal of superoxide radicals that damage the membrane and its function. Catalase is responsible for the detoxification of H_2O_2.

Upon administration, MPTP crosses the blood brain barrier (BBB) and metabolized in astrocytes to its active metabolite 1-methyl-4-phenly pyridinium ion (MPP^+), by monoamine oxidase-B (MAO-B). MPP^+ is selectively taken up by dopaminergic neurons due to its affinity for the dopamine transporter, into cell bodies and projections, followed by transport into the mitochondria and this result in selective toxicity to dopaminergic neurons [15, 26, 27]. Once there, MPP^+ potently inhibits complex I, by inhibiting NADH-ubiuinone oxidoreductase E [28], which poisons the mitochondrial electron transport chain, leading to decrements in cellular ATP and formation of O_2^-. NO combines with O_2^- to form peroxynitrite, which leads to DNA damage.

The antiparkinsonism effect of CT maybe due to the presence of phenols, flavones, triterpenes and glycosides [29]. Different parts of the *C.ternatea* were also found to have high levels of phenolic and antioxidant contents [30]. Several studies have reported that phenolic compounds acts as a potent iron-chelating, anti-inflammatory, nutrigenomic epigenetic antioxidants [31, 32]. The phenolic compound such as alkaloids, saponins, flavonoids, myricetin, coumarins, and lignans [33, 31], present in CT extract may have different functional properties such as scavenging of ROS [34, 12], inhibiting the generation of free radicals and chain-breaking antioxidant activity [35]. They may act as hydrogen-donating radical scavengers by scavenging lipid alkoxyl and peroxyl radical and spares overall antioxidant status [36]. Studies have documented that bioflavanoids and triterpenes exerts free radical scavenging, membrane stabilizing by protecting from lipid peroxidation (LPO), iron-chelating, lipotrophic, vasoactive, immunomodulatory, antimutagenic, XO inhibitory due to the electron and H^+ donating, which is attributed to the free radical quenching and termination of free radical chain reaction based on their reducing power, anti-apoptotic, anti-ageing and neuroprotective effects and increases cytoprotective heat shock proteins [37, 38].

In addition bioflavonoids such as fisetin, quercetin, and rutin are naturally occurring molecules with antioxidant, cytoprotective, and anti-inflammatory actions. It also inhibits the elevation of intracellular calcium [39], which is necessary to convert (XDH) to XO which in turn catalyzes the oxidation of xanthine to provide a source of O_2^- [24] (Olanow, 1993). It has been documented that flavonoids are able to traverse the BBB *in vivo*, however the permeability of certain flavonoids *in vivo* is influenced by their lipophilicity and interactions with efflux transporters [40]. Flavonoids were shown to be potent antioxidants because of their radical-scavenging activity. It was also shown that flavonoids are able to complex heavy metal ions, e.g., iron and copper, which are involved in Fenton-like reactions [41], a protection against DNA strand breaks was demonstrated for quercetin, myricetin, and rutin [42, 43, 44].

Quercetin can scavenge free radicals directly and inhibit the oxidation of various molecules resulting in the activation of antioxidant defense pathways *in vivo* and *in vitro* [45, 46]. Quercetin and its analogs have been established as useful in protecting various neuronal cells against oxidative stress [10, 47].

The aggressive oxidative burden and poor total antioxidant capacity was encountered to be altered on treatment with CT extract enriched with bioflavanoids such as quercetin, kaempferol, and myricetin [34, 48, 49].

CONCLUSION

MPTP induced parkinsonism albino mice were used to prove the antiparkinson effect of *Clitoria ternatea* extract (CTE). MPTP is considered as a precious tool to induce parkinsonism in animals and study the effect of CT. This was concluded using the results obtained from the present study for PARP enzyme assay, XO assay in serum and tissue, TAC in tissue, and supported by histopathological studies. *Clitoria ternatea* was found to increase the total antioxidant capacity (TAC), decrease the XO level in the serum and brain tissue, and reduces PARP enzyme level in brain striatal tissue and serum, and minimize the brain cell death present after MPTP exposure. The

higher dose of CT showed more neuroprotective effect on brain striatal tissue and more effect on PARP enzyme and XO levels in both serum and striatum. The present study implies that the ethanolic extract of *Clitoria ternatea* "Butterfly pea" enriched with triterpenoids, flavonol glycosides, anthocyanins and steroids, has antiparkinsonism effect. The reported activity may be due to the presence of bioflavonoids. Since flavonoids are known to have antioxidant effect, preventing oxidative stress, making complexes with heavy metals, radical–scavenging properties, and preventing the cell death. Further studies may be necessary for identification and characterization of the bioactive compounds responsible for the effect of CT on Parkinson's disease biomarkers.

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