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Effect of the methanol leaves extract of *Clinacanthus nutans* on the activity of acetylcholinesterase in male mice

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ABSTRACT

Objective: To evaluate the *in vivo* effect of 14 d repeatedly oral administration of *Clinacanthus nutans* (*C. nutans*) methanol leaves extract (250 mg/kg, 500 mg/kg and 1 000 mg/kg bw) on the acetylcholinesterase (AChE) activity in male Balb/C mice. **Method:** First group was served as control group, orally treated with distilled water as vehicle and group 2–4 were orally treated with a single daily dose of 250 mg/kg, 500 mg/kg and 1 000 mg/kg bw of *C. nutans* extract, respectively for 14 d. Each group consisted of six animals ($n=6$). The activity of acetylcholinesterase in brain, liver, kidney and heart of mice was determined according to Ellman method (1961). **Results:** From the results obtained, the AChE activity was found to be highest in mice liver, followed by brain, kidney and heart. Methanol extract of *C. nutans* leaves at 250 mg/kg ($P<0.001$), 500 mg/kg ($P<0.001$) and 1 000 mg/kg ($P<0.001$) showed a significant increase in the AChE activity in mice kidney, liver and heart. On the other hand, the AChE activity obtained from the mice brain showed insignificant difference between the control group and treatment group. However, there was no abnormal behavioural change and adverse effect related to the central nervous system observed in all treated mice during 14 d experimentation period. **Conclusion:** In conclusion, 14 d oral administration of *C. nutans* was able to modulate cholinergic neurotransmission by activating AChE activity in mice kidney, liver and heart. Compounds that responsible for the induction of AChE activity in mice liver, heart and kidney and its mechanism needs to be elucidated.

1. Introduction

Malaysia has numerous medicinal plants with high medicinal values. One of the medicinal plants of interest is known as *Clinacanthus nutans* (*C. nutans*) Lindau (Family: Acantheceae) or commonly known as Sabah Snake Grass or Belalai Gajah in Malaysia[1]. In Malaysia, the fresh leaves of this plant are usually boiled with water and consumed as herbal tea. This plant is an important traditional herbal medicine in Indonesia and Thailand for treating skin rashes, insects and snake bites, lesions caused by herpes simplex virus, diabetes mellitus, fever and diuretics[2–3]. Our previous toxicology study has revealed on the safe use after 14 d oral administration of methanol leaves extract of

C. nutans at 300mg/kg, 600 mg/kg and 900 mg/kg in Sprague Dawley female rats without causing liver and kidney injury[4]. In a continuation of our studies focus on the *C. nutans*, we reported herein the *in vivo* biological activity of this plant on the nervous system for the first time.

Cholinesterase is the members of the serine hydrolase family. There are two major types of cholinesterases, *i.e.* acetylcholinesterase and butyrylcholinesterase[5]. Acetylcholinesterase (AChE; EC 3.1.1.7) is the enzyme that terminates the cholinergic nerve transmission by hydrolysing the acetylcholine into choline and acetate[5]. Up to date, there is no study reported on the *in vivo* effect of methanol leaves extract of *C. nutans* on the activity of AChE in laboratory animals. The objective of the present study was to examine the effect after 14 d oral administration of methanol leaves extract of *C. nutans* (250 mg/kg, 500 mg/kg and 1 000 mg/kg) on the activity of AChE in Balb/C male mice liver, kidney, brain and heart.

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2. Materials and methods

2.1. Raw plant and extraction

The fresh leaves of *C. nutans* were purchased from the Herbal Park which is located in Seremban, Negeri Sembilan, Malaysia. All the fresh *C. nutans* leaves were air dried and blended into fine powder. The *C. nutans* extract was prepared using the cold maceration process for 72 h at the room temperature under constant shaking. The extract collected was filtered with filter paper and concentrated in vacuo using rotary evaporator under reduced pressure at 40 °C. The crude methanol extract of *C. nutans* will be freeze-dried in a freeze-dryer at -50 °C until use^[6].

2.2. Animal selection and grouping

A total of 24 healthy young male Balb/C mice ((25±5) g body weight) were used in the present study. All mice were handled according to the Research Ethics guidelines under Faculty of Pharmaceutical Sciences, UCSI University (Proj-FPS-EC-13-001). The animals were kept in an animal holding room under controlled environmental conditions (25±1) °C, air-conditioned, 12 h light and dark cycles^[7]. All animals were allowed to free access to food (standard diet of food pellet) and tap water prior to the start of the study. All mice were randomly assigned into four groups with six animals per group ($n=6$). First group was orally treated with distilled water and served as control group, whereas the remaining 3 treatment groups were orally treated with a single dose daily of 250 mg/kg, 500 mg/kg and 1 000 mg/kg of *C. nutans* extract up to 14 d.

2.3. Preparation of cytosolic fraction from various organs

On day-15, all mice were sacrificed by cervical dislocation. The vital organs such as brain, heart, liver and kidney were removed and washed with the distilled water. Organ

homogenate (1 g organ: 4 mL of 0.1 M phosphate buffer) was prepared by using a homogeniser. Differential centrifugation technique was used to prepare the subcellular organ cytosolic fractions. Firstly, an initial centrifugation at 600 × *g* for 5 min was used to sediment the cell debris, nuclei and unbroken cells. The pellet was discarded and the supernatant was sent for centrifugation at 10 000 rpm for 22 min to sediment the mitochondrial fraction. The supernatant was again centrifuged at 10 000 rpm for 22 min to obtain a clearer cytosolic fraction^[8]. The protein content of each cytosolic fraction from liver, kidney, brain and heart was determined based on Lowry method^[9]. Later, the cytosolic fraction was used as source of acetylcholinesterase for AChE assay.

2.4. Determination of acetylcholinesterase activity

The acetylcholinesterase activity in brain, heart, kidney and liver obtained from mice was determined based on the colorimetric method described by Ellman *et al*^[10]. The reaction mixture consisted of the substrate, 0.5 M acetylthiocholine iodide, 0.3 M DTNB, 0.1 M phosphate buffer at pH 7.4 and 100 µg/mL of organ cytosolic fraction obtained from the control group or those mice treated with different doses of 250 mg/kg, 500 mg/kg and 1 000 mg/kg of methanol leaves extract of *C. nutans*, respectively. The mixture was incubated for 5 min at 37 °C. Approximately 2 mL of the reaction mixture was transferred into curvette and absorbance at 412 nm was recorded by using UV-visible spectrophotometer.

3. Results

Based on the results obtained, no significant changes were seen on the body weight, water and food consumption between the control group and the *C. nutans* treated groups after 14 d treatment (Table 1 & 2 & 3). Repeatedly 14 d oral

Table 1.

Effect of 14 d repeated oral administration of methanol leaves extract of *C. nutans* on the body weight in Balb/C male mice.

Groups(mg/kg)	Body weight (g/mouse/d)			
	Day-0	Day-3	Day-8	Day-14
Control	25.020±1.699	24.800±1.525	25.000±1.456	25.460±1.681
250	24.810±1.852	25.050±2.000	25.150±1.915	25.420±2.123
500	24.630±2.196	25.190±2.188	24.960±2.020	24.490±3.304
1 000	24.940±2.047	25.130±1.673	24.690±1.646	24.430±1.727

$n=6$; Values were expressed in mean±S.D.; Analysed using Dunnett's Test.

Table 2.

Effect of 14 d repeated oral administration of methanol leaves extract of *C. nutans* on the food consumption in Balb/C male mice.

Groups(mg/kg)	Food consumption (g/mouse/d)			
	Day-0	Day-3	Day-8	Day-14
Control	2.620±0.071	2.620±0.307	2.960±0.104	2.500±0.148
250	2.880±0.131	2.940±0.071	2.940±0.072	2.640±0.227
500	2.490±0.055	2.290±0.137	2.860±0.077	2.400±0.016
1000	2.390±0.356	2.340±0.329	2.860±0.049	2.600±0.082

$n=6$; Values were expressed in mean±S.D.; Analysed using Dunnett's Test.

administration of methanol leaves of *C. nutans* extract at 250 mg/kg, 500 mg/kg and 1 000 mg/kg did not cause any significant changes on the relative organ weight between the control and the treatment mice (Table 4). The activity of acetylcholinesterase obtained from the mice liver, kidney and heart at 250 mg/kg ($P<0.01$), 500 mg/kg ($P<0.01$) and 1 000 mg/kg ($P<0.01$) was significantly higher than the control group (Table 5). However, there was no significant difference seen in the activity of brain AChE between the treatment and control group (Table 5).

4. Discussion

Ellman method is a simple and rapid method to determine the activity of acetylcholinesterase. In this reaction, the abstract known as acetylthiocholine iodide is hydrolysed by AChE to liberate one of its metabolites, known as thiocholine. The thiocholine released from this reaction will react with Ellman's reagent, DTNB to form yellow coloured compound (5-thio-2-nitrobenzoate) that can be quantified spectrophotometrically by absorbance at 405 nm^[10]. The absorbance is higher if the activity of AChE is activated.

Based on the results obtained, methanol leaves of *C. nutans* extract was reported to have acetylcholinesterase induction effect in mice liver, kidney and heart. In rodents, the acetylcholinesterase increases in a time-dependent manner after birth and attains its stability at 21 d of neonate

rats^[11]. AChE is mainly synthesised in the hepatocytes and it is distributed to target sites through blood circulation. There are six forms (monomer, dimer, tetramer, tailed tetramer, double tailed tetramer and triple tailed tetramer) of AChE have been characterised^[12]. Liver and heart are containing all types of AChE isoforms above while brain is mainly containing monomer and tetramer and kidney has only monomer and dimer of AChE^[13-15]. Repeatedly 14 days oral administration of *C. nutans* leaves extract at a dose up to 1 000 mg/kg bw was able to deliver sufficient amount of phytochemicals to the liver, kidney and heart via systemic to exert an induction effect on the activity of AChE in male mice.

The phytochemicals in *C. nutans* and its mechanism of action on the positive modulation on the activity of AChE are remained unknown at the moment and it needs to be elucidated. Literature search showed that the flavonoids, saponin and alkaloids were present in the leaves of *C. nutans*^[16]. Flavonoids (*e.g.* C-glycosyl flavones and flavonols) and alkaloids (*e.g.* physostigmine, galantamine) have been scientifically reported to exert an *in vitro* anti-acetylcholinesterase activity^[17-19]. Other than phytochemicals, cortisone and gonadotrophin have been previously reported to induce AChE activity in mice cortex of the thymus and ovarian follicles, respectively^[20,21]. Administration of 1 g/L of caffeine increased the activity of AChE in hippocampal of neonate rats^[22]. Additionally, the expression of AChE is detected at the early stage of

Table 3.

Effect of 14 d repeated oral administration of methanol leaves extract of *C. nutans* on the water consumption in Balb/C male mice.

Groups(mg/kg)	Water consumption (g/mouse/d)			
	Day-0	Day-3	Day-8	Day-14
Control	3.340±0.728	3.330±0.333	3.670±0.367	3.450±0.654
250	3.000±0.361	3.000±0.361	3.170±0.181	3.170±0.181
500	3.170±0.181	3.670±0.367	4.000±0.734	3.000±0.361
1 000	3.500±0.548	3.340±0.728	3.340±0.367	3.340±0.728

n=6; Values were expressed in mean±S.D.; Analysed using Dunnett's Test.

Table 4.

Effect of 14 d repeated oral administration of methanolic leaves extract of *C. nutans* on the relative organ weight in male Balb/C mice.

Groups (mg/kg)	Relative organ weight (g/100 g body weight)					
	Brain	Heart	Kidney	Liver	Lung	Spleen
Control	1.610±0.159	0.490±0.027	1.410±0.129	4.470±0.175	0.600±0.023	0.340±0.039
250	1.540±0.092	0.490±0.067	1.400±0.116	4.530±0.155	0.640±0.060	0.370±0.054
500	1.670±0.200	0.560±0.076	1.480±0.137	4.420±0.193	0.670±0.044	0.400±0.051
1 000	1.610±0.169	0.520±0.058	1.540±0.072	4.420±0.140	0.660±0.055	0.330±0.063

n=6; Values were expressed in mean±S.D.; Analysed using Dunnett's Test.

Table 5.

Effect of 14 d repeated oral administration of methanol leaves extract of *C. nutans* on the activity of acetylcholinesterase in Balb/C male mice.

Grouping (mg/kg)	Acetylcholinesterase activity (μ mol/min/mg)			
	Brain	Heart	Kidney	Liver
Control	2.290±0.414	1.190±0.240	2.100±0.175	5.640±0.102
250	2.700±0.592	3.020±0.347***	2.460±0.120***	9.000±0.213***
500	2.840±0.234	4.360±0.299***	2.690±0.317***	7.730±0.250***
1 000	2.450±0.544	5.440±0.154***	3.140±0.235***	6.760±0.245***

n=6; Values were expressed in mean±S.D.; Analysed using Dunnett's Test. *** $P<0.001$ as compared to control.

apoptosis induced by cyclohexamide and tumour necrosis factor (TNF)[5]. In general, any changes of the activity of AChE will affect the concentration of acetylcholine. Although the activity of AChE was significantly elevated in the mice liver, kidney and heart, but there was no adverse signs related to nervous system was observed during 14 d experimental duration.

The activity of AChE in the mice brain was not affected by the 14 d treatment of *C. nutans* extract. Blood brain barrier (BBB) is formed by brain capillary endothelium. It restricts the diffusion of microscopic objects or hydrophilic molecules and permit the diffusion of hydrophobic compounds. In order to penetrate the BBB, the drug molecules or compound must have high lipid solubility, molecular mass less than 400 Da (g/mol) and the drug forms less than 8–10 hydrogen bonds with solvent water[12]. Based on the phytochemical analysis, many of the bioactive compounds (e.g. orientin, vitexin, β -sitosterol, stigmasterol, lupeol) identified from the *C. nutans* leaves to have molecular weight larger than 400 dalton and hydrophilic in nature[3,23]. Due to this reason, these bioactive compounds could not penetrate across the BBB to exert their effects on AChE activity in mice brain.

In conclusion, repeated 14 d oral administration of methanol leaves extract of *C. nutans* at 250 mg/kg, 500 mg/kg and 1 000 mg/kg was able to induce the activity of AChE in male mice liver, kidney and heart. However, this induction effect was not associated with the appearance of any toxic signs related to neurological disorder in male mice. Phytochemicals in methanol leaves extract of *C. nutans* that responsible for the induction of AChE activity and its possible mechanism of action need to be elucidated in future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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