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In-Vitro Antioxidant and Acetylcholinesterase Inhibitory Activities of *Tradescantia zebrina.*

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ABSTRACT

Tradescantia zebrina pendula (Family: Commenlinaceae) have been commonly used for treatment of various disorders including amenorrhoea, stomachache, hypertension and acts as a diuretic. The objectives of this experiment were to evaluate the in vitro antioxidant and the acetylcholinesterase inhibitory activity of methanol leaves extract of T. zebrina.Total Phenolic content (TPC), Total Flavonoids Content (TFC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of T. zebrina extract were determined using colourimetric methods. The activity of acetylcholinesterase activity was determined based on the method described by Ellman et al(1961) by using acetylcholine iodide as substrate. Based on the results obtained, the methanolic extract of T. zebrina at 100.00ug/ml and 10.00ug/ml showed significant inhibition on the activity of acetycholinesterase ($p<0.05$) of 14.0% and 15.3%, respectivelyas compared to the control group. T. zebrina extract at 0.1 mg/ml exhibited 18.1 % reduction and capability to scavenge free radical against DPPH. In conclusion, methanol leaves extract of T. zebrina exhibited free radical scavenging and acetylcholinesterase inhibitory activities. Further studies are required to isolate the other components in the T. zebrina extract that are possibly responsible for the inhibition of acetylcholinesterase.

Keywords: Tradescantia zebrina; DPPH; Total Flavonoids Content; Total Phenolic Content, Acetylcholinesterase

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INTRODUCTION

Herbal medicine or traditional medicine is a fundamental part of the practice of ancient civilization to improve the health and Malaysia is not an exception.According to the statistic reported by Division of Traditional and Complementary Medicine from Malaysia Ministry of Health (MOH), a total of 55.6% Malaysians make useof traditional and complementary medicine within the past 12 monthsin year 2009 [1]. *Tradescantia zebrina* pendula (Family: Commenlinaceae) or commonly known as purple wandering jew has been widely used for treating amenorrhoea, hypertension, gastrointestinal disorders and gastric cancer [2,3]. *T. zebrina*has glabrous green leaves flushed with purple on the surface and the leaves are usually in oblong-ovate shape [4]. Phytochemicals identified in the *T. zebrina* leaves are including β-sitosterol, succinic acid, zebrinin, anthocyanin, flavonols, apigenin and etc. [5].

Acetylcholinesterase (E.C 3.1.1.7) is a key enzyme present in nervous tissue, muscle and red blood cells that hydrolyses acetylcholine into choline and acetic acid [6]. Acetylcholinesterase (AChE) is present throughout the body, but is particularly important in neuromuscular junctions where it terminates the synaptic transmission and removes the neurotransmitter acetylcholine.

Literature review revealed that β-sitosterol, succinic acid, flavonols and anthocynin have been reported to exhibit *in vitro* anti-cholinesterase activity [7,8]. Hence, we hypothesise the extract of *T. zebrina* leaves is able to inhibit the acetylcholinesterase activity *in vitro* due to the presence of these phytochemicals. Up to date, the *in vitro*anti-cholinesterase activity of *T. zebrina* extract has not been elucidated. The objectives of this study were to determine the *in vitro* antioxidant and acetylcholinesterase inhibitory activities of the methanol extract of *T. zebrina* leaves.

METHODS AND MATERIALS

Chemicals and Reagents

Acetylthiocholine iodide, 5,5'-dithio-bis-nitrobenzoic acid, dipotassium hydrogen phosphate, potassium dihydrogen phosphate and methanol were supplied by local chemical suppliers. All chemicals and solvents used were industrial grade.

Plant Material and Extraction

Fresh leaves of *T. zebrina* were obtained from Ipoh, Perak. The fresh leaves were manually separated from the stalks and air-dried in the in the lab for 7 days. The dried Leaves were ground into dried powder (16.7g) using blender.Powdered dried leaves (16.5g) were macerated with methanol (500 mL) in conical flasks for 3 days at room temperature (25±2°C). The solution was stirred using magnetic stirrer. The conical flasks were covered with aluminium foil to reduce possibility of photo-degradation of the constituents of the extracts. The extraction process was repeated until the process yield transparent filtrates. The mixture was filtered using filter paper (Double Rings, China) to remove insoluble materials and concentrated using rotary evaporator (Büchi, Switzerland) at 40°C. The concentrated crude extract was freeze dried at -50°C using freeze dryer (Christ, Germany) for 24 hour. The freeze-dried extract (1.02 g) was stored in the desiccator filled with silica desiccant to absorb remaining moisture until further usage.

Fourier Transform Infrared (FT-IR) Profiling

Spectra were obtained with Nicolet™ iS5 FTIR spectrometer controlled by OMNIC software for spectra collection and TQ Analyst software for Data processing (Thermo scientific, USA). The instrument is equipped with iD5 ATR accessory featuring a top plate diamond crystal with a fixed angel of incidence of 42°. The freeze-dried extract was used for FT-IR spectroscopy analysis recorded in the range from 4000 to 600 cm^{-1}. A background scan of air was done prior to each scanning of the samples. The spectra were recorded at 4 cm^{-1} resolution with average of 16 scans per spectrum. The diamond crystal of the ATR accessory was cleaned by ethanol before each application of the samples.

Determination of Total Phenolic Content(TPC)

The TPC of the *T. zebrina* extract was measured with Folin-Ciocalteau method described by Brighente et al. (2007) [9]. Briefly, 0.1 ml of *T. zebrina* extract (0.1 mg/ml) was mixed with 0.25 ml of 50% Folin-Ciocalteau reagent and 2 ml of 2% (W/V) sodium carbonate. The final mixture was left for 30 minutes in the dark before reading at 750 nm with spectrophotometer. All measurements were conducted in triplicates and the data were expressed as mg Gallic acid equivalent per g dry weight of extract based on Gallic acid calibration curve. This experiment was repeated in triplicate (n=3).

Determination of Total Flavonoids Content (TFC)

Equal volume of Aluminum chloride (10%) and 0.1 ml of 1 M Potassium acetate (1 M) were added to 0.5 ml of extract (0.1 mg/ml), followed by 2.8 ml of distilled water. The solutions were mixed and incubated at room temperature for 30 minutes before the absorbance was taken at 435 nm [10]. The flavonoid concentration was expressed as mg catechin per g of extract. This experiment was repeated in triplicate (n=3).

Determination of DPPH Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to measure the free radical scavenging activity of *T. zebrina* extract based on the method described by Sharma and Bhat (2009) with slight modification [11]. Ascorbic acid (20µg/ml) was used as positive control in this study. A volume of 1 ml of DPPH solution (1mM) was mixed with 0.5 ml of *T. zebrina* extract (0.1 mg/ml). All samples were mixed using vortex stirrer and incubated in dark for 30 minutes at room temperature. Absorbance was recorded using spectrophotometer at 517 nm. This experiment was repeated in triplicate (n=3).

Determination of Acetylcholinesterase Activity

Differential centrifugation technique was used to prepare liver cytosolic fraction (1 g rat liver: 10ml of phosphate buffer, pH 7.4) as source of acetylcholinesterase. The acetylcholinesterase activity was determined using two-step approach with slight modifications from the standard Ellman method [12]. The reaction mixture consisted of the substrate, acetylthiocholine iodide (15.6 mM), phosphate buffer (pH 8.0) and cytosolic fraction of liver tissue. Five different concentrations of methanol extract of *T. zebrina* leaves ranging from 0.01µg/ml to 100µg/ml were added into the treatment groups. Donepezil (1 µg/ml) was used as positive control in the present study. Equal volume of distilled water (as vehicle) was used to replace the *T. zebrina* extract for the control group. The mixture was incubated for 10 minutes at room temperature. Then, the colouring reagent, DTNB (1mM) was added immediately into each group after 15 minutes of incubation and thoroughly mixed using a vortex stirrer. Absorbance was recorded using spectrophotometer at 412 nm. This experiment was repeated in triplicate (n=3).

Statistical Analysis

Results were presented as mean ± standard deviation.One-way analysis of variance (ANOVA) and a post-hoc, Dunnett's test was carried out to determine significance. $P<0.05$ was considered significant.Analysis was done using GraphPad Prism 5 (GraphPad Sortware, Inc, USA).

RESULTS

The FT-IR spectrum was shown in Figure 1. FT-IR spectrum of the extract showed peaks at 1058.49, 1628.02, 2925.91 and 3396.25 cm^{-1}. Table 1 showed the interpretation of the FT-IR spectrum with reference to the functional groups of the corresponding wavenumbers.The percentage yield of the methanol crude extract, total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity in the methanol extract of *T. zebrina* were shown in the Table 2. TPC was 33.5±2.58 mg Gallic acid equivalent/g and TFC was 9.4±1.06 mg Catechin equivalent/g extract. In the DPPH assay, the scavenging activity measured from the methanol leaves extract of *T. zebrina* leaves was 18.1 %. However, radical scavenging activity of *T. zebrina* was lower compared to the positive control, ascorbic acid (20 µg/ml).

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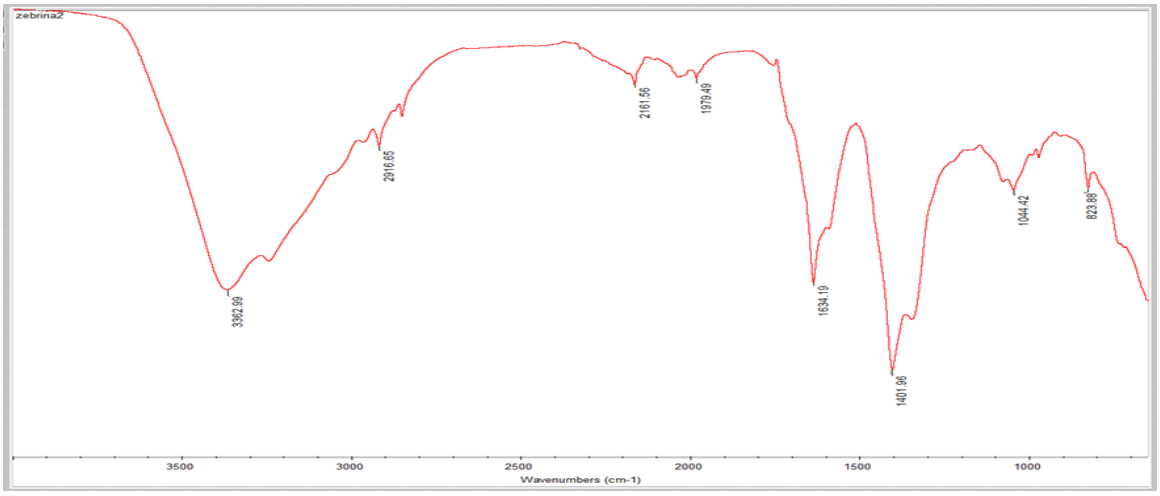

Figure 1. FTIR spectrum of methanol extract of *Tradescantia zebrina* leaves

Table 1.FT-IR spectrum interpretation

Wavenumbers (cm^{-1})	Functional group
1044.42	C-O stretch
1401.96	CH_3 bend
1634.19	Carbonyl (C=O) stretch
2916.65	C-H stretch
3362.99	Hydroxyl (-OH) band

Table 2. Determination of Total Phenolic Content (TPC), Total Flavonoids Content (TFC) and DPPH Radical Scavenging Activity of Methanol Extract of *T. zebrina* Leaves

	% Yield of Crude Extract	TPC (mg GAE/ g extract)	TFC (mg CE/ g extract)	Radical Scavenging Activity (%)
Tradescantiazebrina	6.18	33.5±2.58	9.4±1.06	18.1 ±1.60
Ascorbic Acid				38.1±2.52

N=3; Value = mean ± Standard deviation

Methanol leaves extract of *T. zebrina* was able to inhibit the activity of acetylcholinesterase. As shown in Table 3, the acetylcholinesterase activities in the presence of *T. zebrina* extract at 100 µg/ml (p<0.05) and 10 µg/ml (p<0.05) were significantly reduced 14.0% and 15.3%, respectively compared to the control group.The positive control, donepezil at 1 µg/ml showed a significant inhibition of 17.8 % in comparison to the negative control group.However, *T. zebrina* extract at 1µg/ml, 0.1µg/ml and 0.01µg/ml did not affect the activity of acetylcholinesterase significantly when compared with the control group.

Table 3. *In vitro* Effect of Methanol Leaves Extract of *T. zebrina* on the Activity of Acetylcholinesterase

Concentration of *T. zebrina* extract (µg/ml)	Acetylcholinesterase Activity (mmol/min/mg tissue)
Blank Control	4.78 ± 0.111
Positive Control [Donepezil (1 µg/ml)]	3.92 ± 0.127*
100	4.11 ± 0.168*
10	4.05 ± 0.449*

1	4.92 ± 0.403
0.1	4.69 ± 0.191
0.01	4.68 ± 0.161

N=3; Value = mean ± standard deviation; Results were analysed using post-hoc, Dunnett's Test.
*p<0.05 was considered as significant different compared to blank control.

DISCUSSION

Cholinesterase has been linked to many disorders such as cancer, Alzheimer's disease, cardiovascular diseases and etc. Alzheimer's disease is a chronic, progressive, neurodegenerative disorder of the brain caused by deficit of acetylcholine. Inhibition of brain acetylcholinesterase has always been the major therapeutic target for treating Alzheimer's disease. Galanthamine, rivastigmine and donepezil are considered as the most effective symptomatic treatments for early stages of Alzheimer's disease by inhibiting the acetylcholinesterase [13]. Other than therapeutic purpose, serum cholinesterase is one of the important parameters in the Organisation for Economic Cooperation and Development (OECD) guidelines for the toxicity testing [14]. Many toxic compounds such as organophosphorus pesticides and carbamate pesticides are known to cause cholinesterase inhibition in blood sample.

Livers of the Sprague Dawley rats were used as a source of acetylcholinesterase enzyme in the present study. Acetylcholinesterase is mainly produced in the hepatocytes and circulated to other sites to exert its action. It has been reported that acetylcholinesterase displays a complex molecular polymorphism of quaternary structure [15]. It exists in multiple molecular forms, *i.e*. globular forms consisting of monomer (G1), dimer (G2) and tetramer (G4) while asymmetric forms consisting of tailed tetramer (A4), double tailed tetramer (A8), triple tailed tetramer (A12) [16]. Liver is containing all types of globular and asymmetric forms of acetylcholinesterase. The inhibition of acetylcholinesterase activity by *T. zebrina* extract is expected to prolong the action of Acetylcholine at the nerve system. Based on the present study, there is a possibility of *T. zebrina* extract to inhibit the acetylcholinesterase in the mammalian brain because the acetylcholinesterasein mammalian brain is mainly in G4 form with smaller amounts of G1 form.Donepezil is a piperidine anticholinesterase which specifies in inhibiting the acetylcholinesterase compared to butylrylcholinesterase. The anticholinesterase activity of donepezil at 1 µg/ml in this study was comparable with the effect of *T. zebrina* extract at 10 µg/ml and 100µg/ml on acetylcholinesterase activity. Hence, the *in vivo* study is worthy to be carried out to evaluate the actual effect of *T. zebrina* extract in the brain's acetylcholinesterase activity. Sufficient amount of the active ingredients with anticholinesterase property must enter into the brain to exert its pharmacological action. There are few requirements for phytochemicals to penetrate blood brain barrier. The molecular weight of the phytochemicals must be below 400 Dalton, able to form less than 8-10 hydrogen bonds with solvent water and etc. [17]. Hence, this aspect can only be examined when after the *in vivo* study is done.

The Ellman Method is a simple and reliable method to measure acetylcholinesterase activity in biological samples. In this study, a two-step approach was employed in order to minimise the limitations posed by colouring reagent, DTNB in the experiment. DTNB is prepared in a phosphate buffer of pH 7.0 to ensure the stability of DTNB and the concentration of DTNB is recommended not to be higher than 1.6 mM in working solution [12]. Komersova et al (2007) had reported that the AChE activity measured is lower than the actual results when the concentration of DTNB is far higher than the concentration of substrate [18]. The concentrated DTNB will decrease the hydrolysis rate of substrate in the working solution.

The medicinal properties of plants-derived products are attributed due to the presence of secondary metabolites such as flavonoids, alkaloids and etc. Inhibitory effects of flavonoids on the acetylcholinesterase activity have attracted huge attention among researchers. Selection of solvent for extraction is essential to obtain the targeted phytochemicals for the study. Methanol is one of the commonly used organic solvents to extract phenolic compounds and flavonoids from the plant materials. Based on the phytochemical analysis, the leaves of *T. zebrina* contained phenolic compounds and flavonoids. The FTIR fingerprint of the methanol extract showed a C=O stretching vibration at 1634.19 cm^{-1} and a and at 3362.99 cm^{-1} due to stretching vibration of O-H which indicates the presence of alcoholic carbonyl groups in the structure of the phytochemicals in the extract including the phenolic acids and flavonoids. DPPH assay indicated that methanol leaves extract of *T. zebrina* leaves could act as an electron or hydrogen donator to scavenge radicals. Many

studies had correlated the action of phenolic compounds to anti-oxidative property of the plants [19]. Flavonoids constitute the largest and most important group of phenolic compounds in plants. Thus, flavonoids and phenolic compounds contribute to the antioxidant property exhibited by the methanol leaves extract of *T. zebrina* in this study. Additionally, the results in the present study were in agreement with few studies reported earlier by other researchers whereby anti-cholinesterase activity showed a positive correlation with biologically antioxidant activity, including DPPH assay [20, 21].

CONCLUSION

This present study provides evidence that methanol leaves extract of *T. zebrina* could be a good source of anticholinesterase agent with antioxidant property. Fractionation of the crude extract and *in vivo* acetylcholinesterase inhibitory activity in animal modelare worthy to be carried out in future to confirm its efficacy as potential treatment for neurological disorders such as Alzheimer's disease.

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