

Antioxidant and antiacetylcholinesterase activities of *Pluchea indica* Less.

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Abstract: This study was undertaken to evaluate the antioxidant and acetylcholinesterase inhibition properties of stems and leaves of hexane and methanolic extracts of *Pluchea indica*. Methanolic extract of leaves showed the highest antioxidant activity ($IC_{50} = 24.45 \pm 0.34 \mu\text{g/ml}$) and total phenolic contents ($573.52 \pm 6.2 \text{ mg GAE/100 g}$ crude extract), in DPPH radical scavenging and Folin-Ciocalteu assays respectively, however, it failed to inhibit acetylcholinesterase in TLC bioautographic detection. The rest of plant extracts, including methanolic extract of stems, hexane extract of both leaves and stems, were detected to have acetylcholinesterase inhibitory properties. Hexane extract of both leaves and stems exhibited lower or negligible level of antioxidant activity and phenolic contents. *Pluchea indica* may provide a potential natural source of bioactive compounds, and maybe beneficial to the human health.

Keywords: Total phenolic content, DPPH, antiacetylcholinesterase, *Pluchea indica*

Introduction

Nature has blessed Malaysia with an abundance of varied medicinal plants and currently, Malaysia is among the world's 12 mega biodiversity-rich countries, in terms of number of plant species. More than 20,000 plants species are found in the wild of which 2,000 species has been reported to have medicinal properties (Ang, 2004; Bakri, 2005).

Pluchea indica Less. (Asteraceae) or locally known as *beluntas* is a widespread medicinal plant of Asia, especially India and Malaysia. In Peninsular Malaysia the plant is cultivated in gardens for its young shoots, which can be eaten raw. A decoction of the leaves has been used to combat fever. The sap expressed from leaves is used to treat dysentery. A poultice of leaves is applied externally to treat ulcers and soothe sores (Wiert, 2006). The traditional knowledge and practices on the use of *Pluchea indica* rely exclusively on practical experience and observation passed on verbally from one generation to the next with little documentation. Thus, it is necessary to understand the contemporary relevance of its traditional knowledge using modern methods in the chemical and biological studies of substances.

Herbs or medicinal plants in Malaysia are commonly eaten fresh as a vegetable (salad and *ulam*), especially among the Malay communities.

Most of these herbs are believed to be associated with antioxidant activities and have many beneficial effects (Huda *et al.*, 2007). Many authors have implicated oxygen radicals and other oxygen-derived species as important causative agents in a wide range of disease and disorders. The defenses from endogenous antioxidants are not effective enough to prevent or alleviate diseases and disorders related to oxygen radicals. Therefore, exogenous antioxidants or free radicals scavengers are needed (Zheng and Jia, 1996). At the present time, the most common synthetic antioxidants, such as butylated hydroxy anisole (BHA) and butylated hydroxyl toluene (BHT), propyl gallate (PG) and ter-butyl hydroquinone (TBHQ) are used as antioxidants in fat-containing formulation. However, BHA and BHT have been restricted by legislative rules due to doubts over their toxic and carcinogenic effects (Gülçin, 2006).

Alzheimer's disease (AD) is one of the most common forms of dementia affecting approximately 10% of the population over the age of 65 years (Racchi *et al.*, 2004). In a field of several theoretical options, the best approach has been the use of AChE inhibitors (AChEIs) which led to the introduction of tacrine as the first AChEI specifically approved for the treatment of AD, in 1993 (Whitehouse, 1993). Now, several kinds of AChEIs, such as donepezil, galantamine and rivastigmine are available for the

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symptomatic treatment of patients with mild-to-moderate AD (Racchi *et al.*, 2004). However, these compounds have been reported to have the problems associated with the gastrointestinal disturbances and bioavailability (Schulz, 2003).

Interestingly, intake of polyphenols through diets rich fruits, vegetables and beverages such as red wine was stated to reduce incidence of certain age related neurological disorders including macular degeneration and dementia (Commenges *et al.*, 2000; Bastianetto and Quirion, 2002). Therefore, these evidences suggest that high dietary or supplemental consumption of antioxidants and free radical scavengers may reduce the risk of AD (Mata *et al.*, 2007; Orhan *et al.*, 2007). Thus, this study focused on analysis of total phenolic content, antioxidant and antiacetylcholinesterase activities in the leaves and stems of *P. indica*.

Materials and Methods

Sample collection

The leaves and stems of *Pluchea indica* (L.) were collected from University Agricultural Park (TPU) of Universiti Putra Malaysia.

Sample preparation and extraction

The air-dried, milled leaves of *P. indica* (279.82 g) were macerated in methanol for 72 hrs. The extraction process was repeated three times, the extracts combined and the solvent removed to yield 31.48 g of the crude methanolic extract. The methanol soluble extract (15 g) was triturated with hexane and filtered to give a hexane extract (4.17 g) and a residual methanolic extract (10.22 g). The similar procedure was conducted for the extraction of stems of *P. indica*. Ten gram of methanolic extract of the stems was triturated with hexane and filtered to give a hexane extract (2.83 g) and methanolic extract (7.03 g). Each of the respective extracts was placed in bottle at 4°C until needed.

Determination of total phenolic content

The total phenolic content of each extracts was determined using Folin-Ciocalteu method described by Liu *et al.* (2002), with slight modification. One mg of plant samples (both hexane and methanolic extracts) were dissolved in 10 ml methanol to form 100 ppm of test sample. 500 µl of fractions (triplicates) were taken in test tubes; 0.5 ml of Folin-Ciocalteu reagent and 10 ml of 7% sodium carbonate were added and vortex. All the test tubes were wrapped with dark colored paper. The absorbance of the resulting blue color was measured at 765 nm after one hour.

Quantitative measurements were performed, based on a standard calibration curve of five points: 20, 40, 60, 80, 100 ppm of gallic acid in methanol. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g crude extracts. Blank was prepared by replacing test sample with methanol. All test analysis was run three times in triplicate and averaged.

Determination of DPPH (1,1-diphenyl-2-picrylhydrazyl)

The antioxidant activity of the plant extracts were evaluated on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals according to the previous procedure described by Saha *et al.* (2004). Stock solutions of all the extracts were prepared as 5 mg sample in 1 ml methanol (5000 ppm). The substock solutions (1000 ppm) were prepared from 200 µl stock solutions in 800 µl methanol. The substock solutions were diluted to different concentrations (500 ppm to 7.8125 ppm in methanol) in a 96-well microtiter plate. Then, 5 µl DPPH solutions (prepared as 10 mg DPPH in 4 ml methanol) were added to each well. The plate was shaken gently and placed in the dark for 30 minutes at room temperature. The absorbance was then measured at 517 nm. Percentage inhibition was calculated using the following formula:

$$\frac{\text{OD(DPPH)} - \text{OD(DPPH + sample)}}{\text{OD(DPPH)}} \times 100$$

The IC₅₀ values denoted the concentration of each sample required to give 50% of the optical density shown by the control. All test analysis were run in triplicate and averaged. Quercetin was used as positive control, while methanol was the negative control.

Determination of acetylcholinesterase inhibition properties using TLC method

Anti-acetylcholinesterase activity was measured by using an adaption of the method described by Marston *et al.* (2002). Acetylcholinesterase (EC3.1.1.7) was dissolved in 150 ml of 0.05M Tris-hydrochloric acid buffer at pH 7.8. The stock solution was kept at 4°C. Five milligram of each hexane and methanolic extracts (both leaf and stems) was dissolved in 5 ml of chloroform and methanol, respectively (1000 ppm). Then the prepared samples were spotted on TLC plates. Migration of hexane fractions was conducted with hexane: ethyl acetate (4:1 v/v), while methanol fractions was eluted with varying dilutions using methanol and chloroform (6:4; 5:5; 4:6; 3:7; 2:8; v/v). The dried TLC plates

were sprayed with AChE enzyme and incubated at 37°C for 20 minutes. For detection of the enzymes, solutions of 1-naphthyl acetate (250 mg) in ethanol (100 ml) and Fast Blue B salt (50 mg) in MiliQ water (20 ml) were prepared immediately before use. After incubation of TLC plates, 5 ml of naphthyl acetate solution and 20 ml of Fast Blue B salt solution were mixed and sprayed on the plates to give a purple coloration after 1-2 minutes. The inhibition of AChE was observed from the white spots on the purple colored dye background of the TLC plates.

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS™) software for Windows, Version 16.0 (SPSS Inc., Chicago, IL). Differences in means were determined using ANOVA. Results are expressed as a mean of three determinations \pm SD. Significance level was set as $p < 0.05$.

Results and Discussion

DPPH radical scavenging activity assay

As shown in the Table 1, methanolic leaves extract showed lowest IC₅₀ value which exhibited strong antioxidant activity (IC₅₀ = 24.45 \pm 0.38 μ g/ml). However, this value was lower than those found with the antioxidant standard, quercetin (IC₅₀ = 6.70 \pm 0.79 μ g/ml). Moderate radical scavenging activities was showed by methanolic stems extract (IC₅₀ = 83.74 \pm 0.90 μ g/ml). Both hexane extracts of leaves and stems indicated weak antioxidant activity, with 391.59 \pm 0.59 and 401.68 \pm 0.84 for their IC₅₀ values, respectively. The results showed that polar extracts (methanol) exhibited stronger antioxidant activity than non-polar extracts. The results were in agreement with the results obtained from Nuri *et al.*, 2010 and Vimala *et al.*, 2005.

Table 1. DPPH radical scavenging activity and total phenolic contents of *P. indica* extracts

Samples	IC ₅₀ value (μ g/ml)	Total Phenolic Content (mg GAE/100g crude extract)
Methanol leaves extract	24.45 \pm 0.34	573.52 \pm 6.20
Methanol stems extract	83.74 \pm 0.90	198.14 \pm 2.80
Hexane leaves extract	391.59 \pm 0.59	75.26 \pm 0.94
Hexane stems extract	401.68 \pm 0.83	62.64 \pm 1.85
Quercetin	6.70 \pm 0.78	

The data represent the means \pm SD of triplicate samples of three independent experiments.

TPC assay

The total phenolic content (TPC) values summarized in Table 1 were quantified based on the linear equation obtained from gallic acid standard calibration curve ($y = 0.0074x + 0.0117$; $R^2 = 0.997$). Thus, TPC values were expressed as gallic acid equivalent (mg GAE/100 g crude extracts). From the

table, methanolic leaves extract had highest content of phenolic contents (573.52 \pm 6.20 mg/100 g crude extract), whereas the lowest content was measured in hexane stems extract (62.64 \pm 1.85 mg/100 g crude extract). The results showed that the polarity of the solvent can affect the total phenolic content. Methanolic extract (higher polarity) showed higher total phenolic content than hexane extract. Perez *et al.* (2007) found that methanol was the most efficient solvent as compared to ethanol and water for extracting phenolic compounds. Higher extraction yields of phenolic compounds were obtained with an increase in polarity of the solvent (Cheung *et al.*, 2003). In extracting phenolic compounds from *Gevuina avellana* hulls, Moure *et al.* (2000) suggested that methanol and ethanol were the best extraction solvents compared to acetone. The higher the polarity, the higher the extraction yields of total soluble solids and total extractable polyphenols. Apart from that, water, fat, sugars, proteins, and pigments are the non-phenolics substances that may interrupt the evaluation of TPC. Besides that, Ismail *et al.* (2010) suggested that leaves might be the part that is rich in phenolic compounds in many plants.

Antiacetylcholinesterase activity using TLC method

The AChE inhibition activity was performed after the development of suitable mobile phase solvent system for each extracts and plant parts. Both hexane extracts for both parts showed significant inhibition of AChE in mobile phase solvent system of hexane and ethyl acetate (hexane: ethyl acetate; 8:2; v/v). The white spots were clearly observed on the purple colored dye background of the TLC plates. On the other hand, there was active white spot observed on the methanolic stems extract, while the methanolic leaves extract did not show any activity against AChE. Various mobile phase solvent system (methanol: chloroform; 4:6; 5:5; 6:4; 7:3; 8:2; v/v) were used for the screening. This was due to the occurrences of "tailing" effect (spot with comet-like tail) in the elution of the methanol fraction, and this tailing may affect the observations of white spot formations. The overall results were shown in Table 2. AChE inhibitor is always the target of many Alzheimer dementia drugs (Heinrich and Teoh, 2004). From the study, it was found that the hexane indicated higher anti-AChE activity than methanolic extract. Orhan *et al.* (2007) observed that when polarity increases, the anticholinesterase effects of the plant extracts gradually decreased. This may be most likely due to anti-AChE activity of non-polar compounds found in high amounts within these extracts, which is in accordance with the supposition that the methanolic

extracts, containing the polar compounds, exerted the less inhibitory activity. Houghton *et al.* (2006) reviewed that terpenoids were acetylcholinesterase inhibitor, while there were various studies identified the occurrences of different kind of terpenoids in *P. indica* itself and other *Pluchea* species. This generated the thought that the AChE inhibition properties of *P. indica* extracts could be related to its terpenoids content.

Table 2. AChE inhibition properties of *P. indica* extracts

Extract (1000 ppm)	Part tested	AChE inhibition
Hexane	Leaves	*
	Stems	*
Methanol	Leaves	ND
	Stems	*
Hexane	Leaves	*

Note: * indicated white spot; ND indicated non-detected.

Correlation between antioxidant activity, acetylcholinesterase inhibition properties and total phenolic contents

The results showed that the AChE inhibitory activity was not derived from the same compounds that contributed to the antioxidant activity of *P. indica* extracts. This is because the extract with highest antioxidant activity (methanol leaves) has low detection limit of inhibition on AChE, while the extract that showed significant inhibition on AChE (hexane extracts for both parts) gave low level of free radical scavenging ability. Nevertheless, the methanolic stems extract was found to have both activity of interest. It was detected to have anti-AChE properties and moderate antioxidant power.

The results showed the linear correlation between antioxidant activity and total phenolic content in *P. indica* extracts ($R^2 = 0.703$) suggested that the phenolic compounds contributed significantly to the antioxidant capacity demonstrated by the extracts. Rice-Evans *et al.* (1996), stated that total phenolic compounds have been shown to be responsible for the antioxidant activity of plant material. There are relationship between TPC and scavenging activity (DPPH). It is due to the combined effect of various phenolic compounds and their high hydrogen atom donating abilities (Yan *et al.*, 2010). In addition, the Folin-Ciocalteu assay gives a crude estimate of the TPC present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols (Prior *et al.*, 2005). However, various phenolic compounds respond differently in DPPH assay, depending on the number of phenolic groups they have (Singleton and Rossi, 1965).

Conclusions

The results of this study demonstrated that methanol extracts of stems and leaves exhibited antioxidant activities and contained higher concentration of phenolic compounds, compare to hexane extracts. Relatively, there was linear correlation between the antioxidant activity and total phenolic contents in *P. indica* extracts. With the exception of methanol leaves extract, all the extracts were able to inhibit AChE activity. Methanol stems extract, exhibited both antioxidant and anti-AChE activities. Since the great findings of antioxidant and anti-AChE activities from methanol extracts of *P. indica*, further studies need to be carried out to isolate and identify the bioactive components, especially from stems of *P. indica*.

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