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Original Article

IN VITRO ANTI-ACETYL CHOLINESTERASE AND ANTIOXIDANT ACTIVITY OF SELECTED MALAYSIAN PLANTS

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ABSTRACT

Objective: The aim of this study is to discover and identify the potential plant extracts that could be vital against Alzheimer's disease (AD). It is a neurodegenerative disease affecting the brain. The most promising target for the symptomatic treatment and slowing of AD progression is cholinesterase inhibitors from plants.

Methods: To evaluate the antioxidant and anti-acetylcholinesterase (AChEI) activities of leaves and roots macerated and refluxed with ethanol extracts, from four medicinal plants, namely; *C.asiatica, M.pudica, C.pumila*,and *L.camara.* DPPH and β-carotene assays were used to determine antioxidant activity; whereas Ellman's colorimetric method was adopted to quantify AChEI activity.

Results: Among all tested samples, the refluxed ethanol extract of*M.pudica*leaves exhibited the highest AChEI activity (IC_{50} = 0.0114 mg/ml) whereas, *M. pudica* roots showed high antioxidant activity (DPPH; IC_{50} = 0.0102 mg/ml).

Conclusion: Results reveal that all plant extracts studied possess anti-oxidant properties. Most potent extracts could be a lead to novel antioxidants and acetyl cholinesterase inhibitors, for the treatment of AD.

Keywords: Alzheimer's disease (AD); Acetylcholinesterase activity; Antioxidant activity; β-carotene; DPPH; Medicinal plants.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, named after a German physician, Alois Alzheimer, in 1906 [1]. AD is believed to be the most common cause of senile dementia, of which 50-60% of dementia cases reported in persons over 65 years of age [2]. There are an estimated 35.6 million people living with dementia worldwide and the number is expected to be increased up to 66 million in 2030. Nearly 66% cases of dementia carriers are living in low and middle-income countries [3]. Recent reports on medicinal plants showed that about 63% of low molecular drugs developed from 1981 to 2006, were natural products. These reports suggested that natural products have strong potential to be developed into biologically active compounds with anti-AD activity. Only some natural sources such as *Ginkgo biloba* and *Huperziaserrata* have been studied extensively as natural therapeutic agents to treat AD patients [2].

Four plants namely, *Mimosa pudica*,(Febaceae), *Centella asiatica* (Umbelliferare), *Crotalaria pumila* (Febaceae,) and *Lantana camara* (Verbenaceae), traditionally used in Malaysia for the treatment of neurodegenerative and other common diseases [4], were focused in current study. *C.asiatica* species are used in Malaysia, China, and Indiato treat early nervous and mental illnesses [5]. Whereas mimosan species have been reported to be used for memory-enhancing purposes in Asian folk medicine [6].

Centella, which is from *Centellaasiatica* family, Mackinlayaceae, also known by the synonym *Hydrocotyleasiatica L.*, is used as salad in Malaysia. An ayurvedic formulation composed of four herbs including *C. asiatica,* is assumed to lower the aging effects and prevent dementia, also this herb in conjunction with milk is used for improving memory [7]. The essential oil from *C. asiatica* leaf contains monoterpenoids,bornyl acetate, α-pinene, β-pinene and γ-terpinene are reported as acetylcholinesterase inhibitors. Medicinal values of this plant, suggest that *C. asiatica* may be appropriate to treat symptoms of depression and anxiety in Alzheimer's disease, which may influence cholinergic activity and improving cognitive function. This effect is associated with an antioxidant mechanism in the CNS [5].

The genus *Mimosa* of which *Mimosa pudica*(family Fabaceae) known as chueMue, is a stout straggling prostrate shrubby plant having compound leaves, which are sensitive to touching, pinousstipules and globose pinkish flower heads, grow as weed in almost all parts of the Malaysia. It has been reported that leaves and stems of these plants contain an alkaloid mimosine, whereas leaves also contain mucilage and root contains tannins. *M. pudica* is used as anti-convulsant and cytotoxic properties [8].

Crotalaria species have been extensively used in Chinese medicines. *Crotalaria pumila*(family: Fabaceae) is member of the genus *Crotalaria*. *Crotalaria pumila* is used to treat yellow fever and skin rashes in USA. All plant parts of *C. sessiliflora*Vatke., *C. assamica*Benth., and *C. ferruginea* are used traditionally in China to treat cancer. Aerial parts of *C.agatiflora*Schweinf are used in Kenya for the treatment of ortitis media, bacterial infection of ears, as well as for treating sexually transmitted diseases [9].

Lantanacamara belongs to, the genus *Lantana*(family: Verbenaceae), is commonly known as wild sage. It is the widest spread species of this genus, used as nutritious weed and as a popular ornamental plant [10]. It is listed as one of the most important medicinal plant of the world, *Lantana* plant has been reported to possess a number of medicinal properties [11]. Various parts of the plant are used in the treatment of fistula, pustules, rheumatism, itches, cuts, ulcers, swellings, bilious fever, catarrh, eczema, dysentery and chest in infection of children [12]. Some metabolites isolated from their leaves have antitumor activity, antimotality, anti-inflammatory, insecticidal, germicidal effects and antioxidant activity [13].

Many plants contain natural antioxidants, act in metabolic response to endogenous production of free radicals and other oxidant species.

These responses are due to ecological stress or promoted by toxins produced by pathogenic fungi and bacteria [14]. Recently, interest in naturally-occurring anti-oxidants has been increased, which can protect human beings from oxidative stress [15]. Modern life style and environmental pollution is the major cause of over production of reactive oxygen species (ROS) [16].

Nature is a rich source of biological and chemical diversity. The unique and complex structure of natural products cannot be obtained easily by chemical synthesis. A number of plants are used as traditional medicine in Malaysia, for the study of drugs related to AD. Therefore, the aim of this study is to evaluate *M. pudica, C. Asiatica, C. pumila,* and *L. camara* which have been used to prevent certain kind of dementia. All the plants were selected and screened for their antioxidant and anti-acetylcholinesterase inhibitory activities.

MATERIAL AND METHODS

Plant Material

M. pudica, C. asiatica, C. pumila, and L. camara were collected from the Pahang (East Coast of Malaysia) in March and April 2012. All plants were authenticated by Institute of Biosciences, Biodiversity Unit, and vouchers of specimen were submitted to the herbarium of University Putra Malaysia, Serdang, and Selangor, Malaysia.

Preparation of sample

The dried samples were cut into small pieces and ground into fine powder using a dry grinder. The grinded samples were sieved to get uniform particle size then kept in air tight container and stored around -20 degree Celsius, until further analysis.

Extraction

Plant material was dried in shadow and ground to powder for extraction. The extracts were prepared by maceration, 5g of each plant powder in 30 mL of ethanol (EtOH) for 6 hrs. The macerate was obtained by evaporating the sample in fume hood. The Soxhlet extraction was done by using same ratio of solvent and sample (10 g in 60 mL) and extracted for 6 hrs. An aliquot of each extract was evaporated to obtain the dry weight.

Reagents and Chemicals

All the chemicals of analytical grade were used. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, b-carotene, 2,6-ditert-butyl-4-hydroxytoluene (BHT), acetylcholinesterase (AChE) type VI-S, from electric eel (349 U/mg solid, 411 U/mg protein), 5,5-dithiobis[2-nitrobenzoic acid] (DTNB),positive control Physostigmine (Eserine), Substrate acetylthiocholine iodide (AChI), solvent ethanol (EtOH) and Tween 40, were supplied from Sigma Aldrich, Germany.

Buffers

Four buffers were used. Buffer A: 50mM Tris-HCl, pH 8; buffer B: 50mM Tris-HCl, pH 8, containing 0-1% bovine serum albumin (BSA), buffer C: 50mMtris-HCl, pH 8, containing 0.1M NaCl and 0.2 M $MgCl_2.6H_2O$; and buffer D: 50mM NaH_2PO_4 - Na_2HPO_4, pH 7.6.

ANTI-ACETYLCHOLINESTERASE ASSAY

The enzymatic activity was determined by Ellman's methods as described by Ingkaninan et al. (2003) [17]. Briefly, the enzyme hydrolyzes the substrate acetylthiocholinesterase (ATCI) and produces the thiocholine. Which reacts with Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce 2-nitobenzoate-5-maeceptothiocholine and 5-thio-2-nitrobenzoate, that can be detected at 405 nm. In the 96 well plate, 25 µL of 15 mM ATCI dissolved in water, 125 µL of3 mM DTNB dissolved in buffer C, 50 µL of buffer B and 25 µL of sample (10 mg/mL in ethanol diluted 10 times with buffer A, to give a concentration of 1 mg/mL) were added. Absorbance was recorded at 405 nm for every 45 seconds, for five times. The reading was taken before adding the 25 µL of 0.25 U/mL of enzyme and again absorbance was read after 50 seconds for eight times.

TLC bio autographic assay

Acetylcholinesterase (500U) was dissolved in 500 mL Tris-hydrocholric buffer (0.05 mol/L, pH 7.8) with 500 mg BSA added to stabilize the enzyme during the bioassay. 1-nephtyle acetate (150 mg) was dissolved in ethanol (40 mL) and then diluted by distilled water (100 mL). Samples were applied to silica gel on TLC plate and migrated by using Chloroform: Methanol (7:3) as solvent system. The plate was then dried absolutely using a blow dryer. The enzyme and 1-nephthyl acetate solution were sprayed to TLC plate subsequently. After each spray, TLC plate was blown quickly with cold wind from a blow dryer until no free liquid was present on its surface. Plate was managed to be in moisture, in case of drying, enzyme can be deactivated. However ethanol was removed so that it can't inhibit AChE activity. Plate was kept in a close vessel containing a small amount of water in the form of vapors to avoid any direct contact with water. The plate was kept in the close container at 37 ^{0}C for 20 min to allow the enzyme to react with 1-nephthyl acetate completely. Afterwards, the solution of fast blue B salt was sprayed on TLC plate; the inhibited AChE spots appeared white while other parts turned purple.

ANTIOXIDANT PROPERTIES ANALYSIS

The antioxidant activity was measured by the β-carotene/linoleic acid assay, as described by [18, 19].

1, 2-diphenyl-2-picryl hydrazyl radical (DPPH)

Initial volume 0.1 mL of various concentrations of samples was mixed with 0.4 mL of 0.3 mM DPPH reagent prepared in methanol. The mixture was shaken thoroughly and incubated in the darkness at room temperature for 30 min. The absorbance of the reaction was measured spectrophotometrically at 517nm, immediately after mixing and then after incubation as well. The scavenging effect of DPPH free radical was calculated by using the following equation.

$$Scavenging\ effect\ (\%) = 1 - \left[\left(\frac{Absorbance\ sample}{Absorbance\ control} \right) \times 100 \right] \quad .....(1)$$

Where control is the absorbance of the blank (a reaction with all the reagents except the test extract), and absorbance of sample is the absorbance of the test extract. Tests were carried out in triplicates to obtain 50% inhibition (IC_{50})

β-Carotene bleaching assay

β-Carotene solution was prepared by dissolving 0.2mg of β-carotene in 1mL chloroform, in the round bottom flask containing 0.02mL linoleic acid and 0.2mL 100% Tween 20. To remove chloroform, the mixture was kept at 40 ^{o}C for 10 min by using rotary evaporator. A 100mL of distilled water was added with vigorous shaking to form an emulsion; 5mL of the emulsion was taken out and transferred into test tubes containing 0.2mL of sample. All test tubes were placed in water bath at 45 ^{o}C for 2 hrs. The absorbance of samples was measured at 470 nm using spectrophotometer at initial time (t = 0) against a blank consisting of emulsion without β-carotene. A standard (BHA) at concentration of 1mg/mL was used. The measurement was carried out at 10, 20, 40, 70, 110, and final time is 200 min.

Statistical Analysis

All determinations were done in triplicates, and the results reported as mean ± standard deviation (S.D.). Calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc).

RESULTS AND DISCUSSION

Acetylcholinesterase Inhibition Assay

The results of the AChE inhibitory activities of the tested plant's leaves and roots extracts are illusterated in Figures 1 and 2

A comparetive analysis was made between our examined plants inhibitory actevities and positive control eserine(physostigmin). We found that all the examined plant's extracts posses inhibitory activity against AChE. Ethanol was used as a solvent and it gives better activityas compared to water described in various reportes [20]. The *C. mimosoides*roots showed the highest percentage inhibition of AChE. The higher activity of the Ethanolic extracts might suggest that organic solvents are able to extract more active compounds with possible AChE inhibitory activity than water [21].

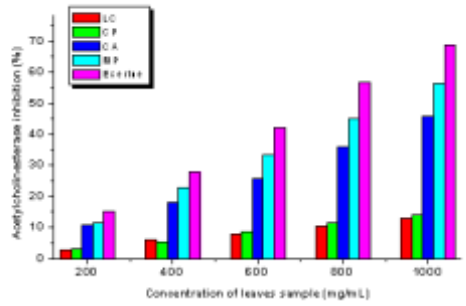


Fig.1: Inhibition of Acetylcholinesterase by *L.camara* (LC), *C.pamula* (CP), *C.asitica*(CA), and *M.pudica(MP)* leaves extracts.

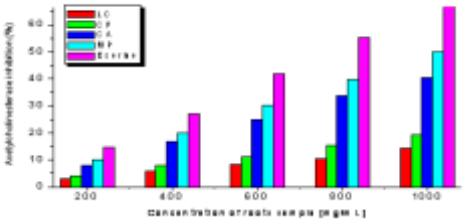


Fig.2: Inhibition of Acetylcholinesterase by *L.camara* (LC), *C.pamula* (CP), *C. asitica*(CA), and *M. pudica*(MP)roots extracts

The IC_{50} values of the plant extracts indicating AChE inhibitory activity, are presented in Table1.

Table 1: IC_{50} values of plant extracts for AChEI activity

Plant	AChE IC_{50} mg/mL	
	Leaves	Roots
M. pudica	0.0114±0.012	0.0168±0.011
C. asiatica	0.0152±0.011	0.0199±0.012
C. pumila	0.0451±0.003	0.0420±0.002
L. camara	0.056±0.011	0.047±0.003

The low IC_{50} value indicates good inhibition of the enzyme. The ethanolic extracts of *M.pudica* leaves has lowest IC_{50} value (0.0114 mg/mL) while its root's extracts activity was recorded 0.0168 mg/mL, indicating that leaves are the best inhibitor of enzyme. As *C.asiatica* leaves are more significant for improving memory as compare to roots, our study proved it after getting IC_{50} of roots (0.0199 mg/mL), for leaves IC_{50} (0.0152 mg/mL). For*C.pumila* both parts showed almost similar results (0.045, 0.0420 mg/mL) for leaves and roots respectivly. On the other hand, *L.camara's* roots inhibit enzyme more (0.047 mg/mL) than the leaves (0.056 mg/mL). It showes that *L.camara's* roots contain polar compounds which dissolved in polar solvent easily as compare to the compounds present in leaves [22].

ANTIOXIDANT ACTIVITIES

DPPH radical scavenging activity

A number of studies show that oxidative stress is involved in age-related neurodegenerative diseases. Likewise, there are numerious studies which have examined the positive benefits of antioxidants to reduce or block neuronal death occurring in the pathophysiology of these disorders [23]. In addition, the antioxidant potential of a compound can be attributed to its radical scavenging ability and total antioxidant activity. In order to determine the ability of the plant extracts to serve as antioxidants, two activities were measured; ability to scavenge DPPH and β-carotene bleaching assay. Figures 3 and 4 present the dose-dependent DPPH radical scavenging activity of the plant extracts.

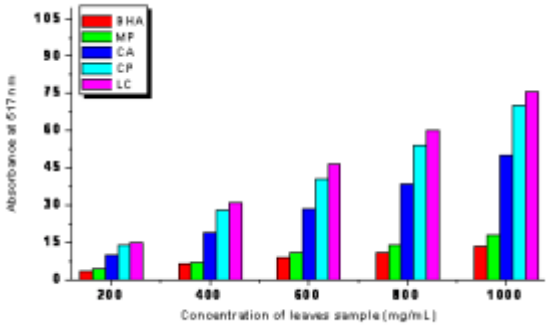


Fig.3: Scavenging activity of ethanolic extracts of *L. camara*(LC), *C. pamula* (CP), *C.asitica*(CA)and *M.pudica*(MP) leaves on DPPH radical.

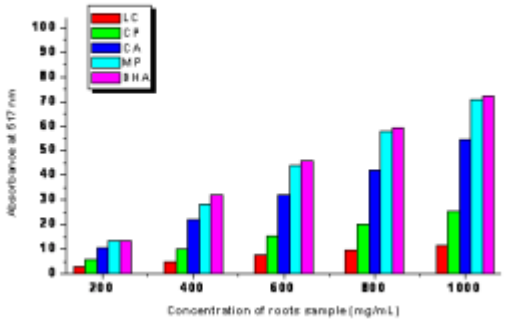


Fig.4: Scavenging activity of ethanolic extract of *L.camara* (LC), *C.pamula* (CP), *C.asitica*(CA)and *M.pudica*(MP) rootson DPPH radical.

In this study, all the plant extracts exhibited high antioxidant potency in terms of electron or hydrogen atom-donating capacity. The IC_{50} values of DPPH as shown in Table 2, depict that the trend of DPPH activity is almost same as AChE activity except for the *L.camara*, whose roots have higher IC_{50} value (0.044 mg/mL) as compare to the leaves (0.0388 mg/mL).

Table2: IC_{50} values of plant extracts for antioxidant activity

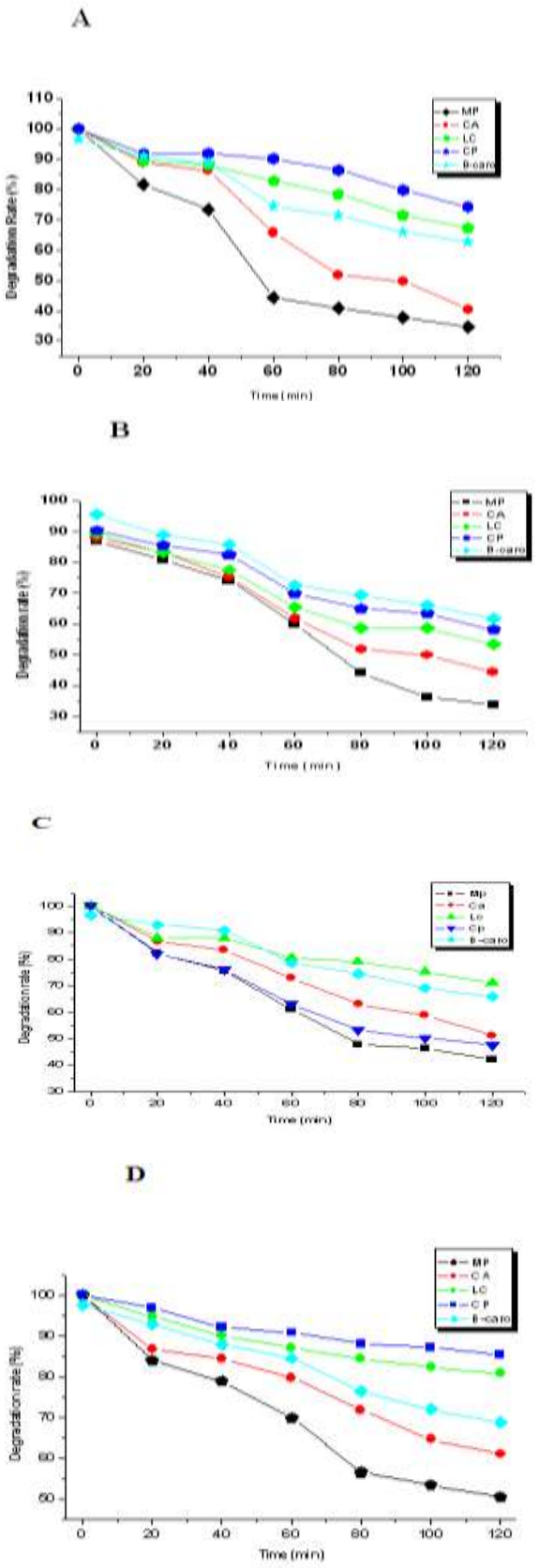
Plant	DPPH IC_{50} mg/mL	
	Leaves	Roots
M. pudica	0.014±0.034	0.0102±0.021
C. asiatica	0.0192±0.080	0.0107±0.013
C. pumila	0.0218±0.001	0.0216±0.021
L. camara	0.0388±0.077	0.044±0.011

β–Carotene Bleaching Assay

According to Wang et al. (2009) the mechanism of beta-carotene bleaching assay is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid by air oxidation [24]. The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radicals formed from linoleic acid. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, that attacks the highly unsaturated beta-carotene molecules. As beta-carotene molecules lose their double bonds by oxidation in this model system, in the absence of an antioxidant, the compound loses

its chromophore and characteristic orange colour, which can be spectrophotometrically monitored.

All the plants extracts studied, possess anti-oxidant properties as measured by β-carotene method (Figure: 5 A-E)



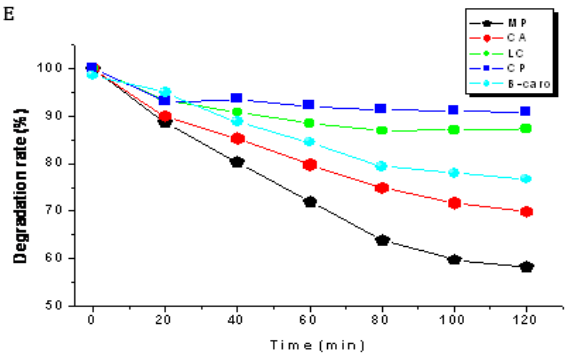


Fig.5: Mean total antioxidant activity of plant ethanolic extracts.
Antioxidant activity was measured using a β-carotene bleaching assay. Where A= 100, B= 250, C= 500, D= 750 and E= 1000 ppm.

The extract of *M.pudica* has the highest percentage of inhibition of oxidation of beta-carotene among all the extracts (Figure 5). The leave extract of *L.camara* has the lowest antioxidant activity according to β-carotene method (Figure 5). Ethanolic extract of all samplesdisplayed anti-oxidant properties similar to those of the anti-oxidant standard BHA, though for higher concentrations as shown in Figure 3

CONCLUSION

Among four Malaysian medicinal plants analysed, all showed inhibitory activity against the enzyme acetylcholinesterase and they also proved to be potent antioxidant. *M.pudica* and *C.asiatica* showed high values for both assays. The ethanolic extract of *M.pudica* roots showed the best inhibition of AChE and a very good antioxidant activity as well. *C.pumila* also proved to be a good inhibitor of acetyl cholinesterase whereas, *L.camara* inhibited to less extent. *M. Pudica* and *C.asiatica* may help in preventing AD as they showed inhibitory activity against AChE and at the same time both bear good antioxidant potency. Finally, these plant extracts and their active components could emerge as natural antioxidants, alternative anticholinesterase drugs and can serve as starting points for synthesizing more effective AChE inhibitors.

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