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ORIGINAL ARTICLE

In vitro acetylcholinesterase inhibitory activity and the antioxidant properties of *Aegle marmelos* leaf extract: implications for the treatment of Alzheimer's disease

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Abstract

Background: Alzheimer's disease (AD) is a progressive neurodegenerative disorder clinically characterized by loss of memory and cognition. The effective therapeutic options for AD are limited and thus there is a demand for new drugs. *Aegle marmelos* (Linn.) (*A. marmelos*) leaves have been used in traditional medicine to promote intellect and enhance memory. In this study, we evaluated *A. marmelos* for its acetylcholinesterase (AChE) inhibitory activity and antioxidant property *in vitro* in the treatment of AD.

Methods: A crude methanol extract and four fractions (petroleum ether, chloroform, ethyl acetate and aqueous) were prepared from the leaves of *A. marmelos*. The preparations were assessed for AChE inhibitory activity by the Ellman method, and their antioxidant properties were assessed by several assays: reducing power, scavenging of 1,1-diphenyl-2-picrylhydrazyl free radical and hydroxyl radical, and inhibition of lipid peroxidation. Qualitative and quantitative analyses of endogenous substances in *A. marmelos* were performed by the standard phytochemical methods.

Results: Among the different extracts tested, the ethyl acetate fraction exhibited the highest inhibition of AChE activity. In the same way, ethyl acetate fraction showed the highest reducing activity and radical scavenging ability towards the 1,1-diphenyl-2-picrylhydrazyl (half maximal inhibitory concentration = 3.84 μg/mL) and hydroxyl free radicals (half maximal inhibitory concentration = 5.68 μg/mL). The antiradical activity of the ethyl acetate fraction appeared to be similar to that of the reference standard butylated hydroxytoluene and catechin used in this study. In addition, the ethyl acetate fraction displayed higher inhibition of brain lipid peroxidation. Phytochemical screening of different extractives of *A. marmelos* showed the presence of phenols and flavonoids, alkaloid, saponin, glycoside, tannin and steroids. Quantitative analysis revealed higher contents of phenolics (58.79-mg gallic acid equivalent/g dried extract) and flavonoids (375.73-mg gallic acid equivalent/g dried extract) in the ethyl acetate fraction.

Conclusion: The results suggest that the ethyl acetate fraction of *A. marmelos* is a significant source of polyphenolic compounds with potential AChE inhibitory property and antioxidant activity and, thus, may be useful in the treatment of AD.

Key words: *acetylcholinesterase inhibition,* Aegle marmelos*, Alzheimer's disease, antioxidant activity, phytochemical screening.*

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INTRODUCTION

Alzheimer's disease (AD), a major cause of mortality in elderly people, is a progressive neurodegenerative disorder characterized by a gradual loss of memory, cognition and behavioural abnormalities. The pathological hallmarks of AD are profound loss of cholinergic neurons, senile plaque consisting of Abeta protein, and neurofibrillary tangles of microtubule-associated protein tau.[1–5] Although there has been tremendous progress in understanding the aetiology and pathogenesis of AD, effective drugs remain limited.

Extensive loss of cholinergic neurons, particularly in the basal forebrain is a prominent feature observed in AD patients that is accompanied by deficiency of acetylcholine (ACh), a neurotransmitter found in the synapses of the cerebral cortex.[6–8] The loss of cholinergic neurons and the associated decrease in levels of ACh has been found to correlate well with the cognitive impairment seen in AD patients.[9] Therefore, elevating ACh by inhibiting acetylcholinesterase (AChE), which is involved in the breakdown of ACh, appears to improve the symptoms of cognitive deficit in AD and serves as an important strategy in the development of drugs. To date, only three cholinesterase inhibitors, donepezil, galantamine and rivastigmine, have been approved by the US Food and Drug Administration to treat AD.[10,11] These drugs have ameliorated symptoms and improved the functioning of patients with AD, but none can completely restrict or reverse the progression of AD.[12]

A substantial body of evidence implicates oxidative stress in the aetiology and pathogenesis of AD. It has been shown that the reactive oxygen species (ROS) and other free radicals, which are formed and accumulated during oxidative stress as a result of an imbalance between their production and removal by the antioxidant system, induce cellular and molecular abnormalities in sporadic AD.[13–16] Although the exact mechanisms underlying these deleterious effects remain unclear, it is known that oxidative stress occurs before the formation of neurofibrillary tangles and senile plaques, both of which are hallmarks of AD.[17,18] It has been further demonstrated that Abeta protein, the major component of senile plaque in the brain of AD patients, causes an increase in free radical production in neuronal cells, leading to oxidative stress and cell death.[19,20] Therefore, antioxidants have been suggested as therapies to prevent, delay or ameliorate the pathological changes underlying the progression of AD.[21,22] Recently, there has been increasing interest in the natural antioxidants contained in the medicinal plants, which are candidates to prevent oxidative damage.[23,24]

Aegle marmelos (*A. marmelos*), commonly known as bael and belonging to the family Rutaceae, is a tree widely distributed throughout Bangladesh. The medicinal properties of *A. marmelos* are well described in Ayurveda, traditional Indian medicine.[25] The leaf of this plant has a folkloric reputation for promoting intellect and enhancing memory.[26] Traditionally, the plant has been used to treat fever, diabetes, diarrhoea, abscesses and snake bites. Phytochemical investigations of *A. marmelos* demonstrated several active elements including marmelosin, marmelide, luvangetin, auraptene, psoralen and tannin.[27,28] The extract of the plant has been reported to possess important pharmacological effects including anti-diabetic, anti-hyperlipidaemic, contraceptive, antidiarrhoeal, analgesic, antipyretic and anti-inflammatory, antimicrobial and anti-proliferative effects.[28–34] A preliminary study has shown that *A. marmelos* has 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.[29]

Although *A. marmelos* has important medicinal values for the treatment of AD, no studies have yet examined its anti-AD capabilities. Therefore, the objective of this study was to evaluate the inhibition of AChE activity and antioxidant properties of *A. marmelos* leaves in order to treat AD.

MATERIALS AND METHODS

Chemicals

Aluminium chloride, ammonium molybdate, ascorbic acid (AA), bicinchoninic acid, DPPH, Folin–Ciocalteu reagent, Tris-HCl and Triton X-100 were obtained from Sigma-Aldrich (Bangalore, India). Gallic acid was obtained from Wako Pure Chemical Company Ltd (Osaka, Japan). 2-deoxy-D-ribose, thiobarbituric acid, (+)-catechin, 5,5′-dithio-bis-(2-nitro) benzoic acid, acetylthiocholine iodide and donepezil were obtained from Sigma-Aldrich (Tokyo, Japan). Unless otherwise specified, all other chemicals were of analytical grade.

Plant materials

The leaves of *A. Marmelos* were collected from the city of Rajshahi, Bangladesh, and identified by an expert taxonomist. A voucher specimen was submitted to the

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herbarium of the Department of Botany, Rajshahi University.

Extraction

The leaves of *A. marmelos* were dried at room temperature for 9 days, finely powdered and used for extraction. Powdered leaves (500 g) were placed in an amber-coloured reagent bottle and soaked in 1.5-L methanol. The contents were sealed in the bottle for 7 days and occasionally shaken and stirred. The whole mixture was filtered through cotton and then through Whatman No. 1 filters paper, and the filtrate was concentrated with a rotary evaporator under reduced pressure at 50°C to obtain the crude methanol extract (CME) (13.485 g). An aliquot (10 g) of the concentrated methanol extract was fractionated as described previously,[35] and the resultant soluble fractions of petroleum ether (PEF, 2.693 g), chloroform (CLF, 1.834 g), ethyl acetate (EAF, 1.580 g) and aqueous (AQF, 6.195 g) were obtained for the experiment.

Phytochemical screening of the plant extract

The extracts were tested to determine the presence of various phytochemicals, including tannins, flavonoids, alkaloids, saponins, glycosides and steroids in accordance with the methods described.[35]

Determination of total phenolic content

Total phenolic content of the different extracts from *A. marmelos* was determined with the Folin–Ciocalteu reagent.[36] The reaction mixture contained 0.5-mL plant extract or standard solution at different concentrations, 2.5-mL Folin–Ciocalteu reagent (diluted 10 times with water) and 2.5-m sodium carbonate solution (7.5%). The test tube was incubated for 20 min at 25°C to complete the reaction, and the absorbance of the reaction mixture was measured at 760 nm. Gallic acid was used as the standard and the results were expressed as milligrams of gallic acid equivalent (GAE)/g of dried extractives.

Determination of total flavonoid content

Total flavonoid content of the different *A. marmelos* extracts was determined by the aluminium chloride colorimetric method.[37] The plant extract (1.0 mL) was added to 3.0-mL methanol, 0.2-mL 10% $AlCl_3$, 0.2-mL 1 M potassium acetate and 5.6-mL distilled water. The test tube was then incubated at room temperature for 30 min to complete the reaction. The absorbance of the solution was measured at 420 nm. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalent (GAE)/g of dried extractives.

Determination of reducing power

The reducing power of different *A. marmelos* extracts was evaluated using the method employed by Oyaizu.[38] Various concentrations of plant extract or standard solutions (1 mL) were mixed with 2.5-mL potassium buffer (0.2 M) and 2.5-mL potassium ferricyanide. After heating for 20 min at 50°C, 2.5-mL trichloroacetic acid (10%) solution was added to the test tube. The total mixture was centrifuged at 3300 *g* for 10 min. Next, 2.5-mL supernatant solution was withdrawn from the mixture and mixed with 2.5-mL distilled water and 0.5-mL ferric chloride (0.1%) solution. The absorbance was measured at 700 nm. AA was used for comparison.

Determination of DPPH radical scavenging activity

DPPH radical scavenging activity of the different *A. marmelos* extracts was determined according to the method reported by Choi *et al*. with slight modifications: 2-mL methanol solution of plant extract or reference standard butylated hydroxytoluene at different concentration was mixed with 3-mL methanol solution of DPPH in the test tube.[39] The test tube was incubated at room temperature for 30 min in a dark place to complete the reaction. The absorbance of the solution was measured at 517 nm. DPPH free radical scavenging ability (%) was calculated with the following formula:

$$((A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}) \times 100$$

Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of different *A. marmelos* extracts was determined by the method described by Elizabeth *et al*. but with a slight modification.[40] The assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). In a final volume of 1 mL, the reaction mixture contained 2-deoxy-2-ribose (2.8 mM);

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KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM); and various concentrations of the test sample or reference compound catechin (CA). After incubation for 1 h at 37°C, 0.5-mL reaction mixture was added to 1-mL 2.8% trichloroacetic acid, then 1-mL 1% aqueous thiobarbituric acid was added, and the mixture was incubated at 90°C for 15 min to develop the colour. After it cooled, the mixture's absorbance was measured at 532 nm against an appropriate blank solution. Hydroxyl radical scavenging ability (%) was calculated by using the formula:

$$((A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}) \times 100$$

Determination of lipid peroxidation inhibition activity

The inhibition of lipid peroxidation activity was determined according to the method described by Liu *et al*. with a slight modification.[41] The 150-g adult long Evan rats were anaesthetized with sodium phenobarbitone. The rats' brain were dissected and homogenized with a homogenizer in ice-cold phosphate buffer (50 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was used as liposome for an *in vitro* lipid peroxidation assay. The ability of plant extract to inhibit lipid peroxidation was studied by incubating rat brain homogenates treated with hydrogen peroxide (10 μM) and different concentrations of plant extract. Hydrogen peroxide induced lipid peroxidation in the rat brain homogenates. Lipid peroxides reacted with thiobarbituric acid to form a pink product, thiobarbituric acid reacting substances, measurable colorimetrically at 532 nm. The difference between the control and plant extract treated sample was the measured decrease in thiobarbituric acid reacting substances formation, reflecting reduced hydroxyl radical-induced lipid peroxidation. CA was used as the reference standard for comparison.

Determination of AChE inhibitory activity

The AChE inhibitory assay was performed according to the colorimetric method by Ellman *et al*.,[42] with acetylthiocholine iodide as a substrate. For the enzyme source, the rat brains were homogenized in a homogenizer with five volumes of a homogenation buffer (10 mM Tris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100), and centrifuged at 10 000 *g* for 30 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Protein concentration was determined by using a bicinchoninic acid kit (Sigma Co., St. Louis, MO, USA) with bovine serum albumin as a protein standard. The rates of hydrolysis by AChE were monitored spectrophotometrically. Each extract or standard (500 μL) was mixed with an enzyme solution (500 μL) and incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding Ellman's reaction mixture (3.5-mL 0.5 mM acetylthiocholine, 1 mM 5, 5′-dithio-bis (2-nitro benzoic acid)) in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme. Donepezil was used as a positive control. The percentage inhibition of AChE activity was calculated using the following formula:

$$((A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}) \times 100$$

Statistical analysis

All analyses were carried out in triplicate. Data were presented as mean ± SD. Free R-software version 2.15.1 (http://www.r-project.org/) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Significant differences (P-value <0.05) between the means were determined using the t-test.

RESULTS

AChE inhibitory activity

Reduction of ACh in the hippocampus and cortex of the brain is one of the most important remarkable changes observed in AD.[5–8] Therefore, elevation of ACh level in the synaptic cleft by inhibition of AChE, which is involved in the breakdown of ACh, is an accepted therapeutic strategy for AD. The inhibitory activity of CME derived from *A. marmelos* against rat brain AChE was determined by Ellman's method.[41] This method estimates AChE using acetylthiocholine iodide (substrate) and dithiobis nitro benzoic acid. The enzymatic activity was measured by the yellow colour compound produced by thiocholine when it reacts with dithiobis nitrobenzoate ion. The result of the

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Table 1 AChE inhibitory activity of different extractives from *Aegle marmelos*

Concentration (μg/mL)	AChE Inhibition %)					
	CME	PEF	CLF	EAF	AQF	Donepezil
50	16.20 ± 2.41[c]	8.75 ± 1.40[a]	13.48 ± 1.75[b]	23.68 ± 1.63[e]	17.71 ± 1.49[d]	92.76 ± 2.15[f]
100	29.55 ± 1.72[c]	14.07 ± 1.31[a]	22.06 ± 1.57[b]	36.95 ± 0.97[e]	31.46 ± 2.00[d]	97.14 ± 0.99[f]
200	35.78 ± 1.56[c]	20.37 ± 1.36[a]	29.87 ± 1.84[b]	47.58 ± 1.57[e]	40.13 ± 1.74[d]	98.58 ± 1.43[f]

AChE, acetylcholinesterase; AQF, aqueous fraction; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; PEF, petroleum ether fraction. [a–f]Means between columns differ significantly ($P < 0.05$) in each concentration.

AChE inhibitory activity of CME is shown in Table 1. The extract showed AChE inhibitory activity in a concentration-dependent manner. CME was found to inhibit AChE activity by 29% at a concentration of 100 μg/mL, while donepezil, used as the reference standard in this study, inhibited the AChE activity by 97% under the same experimental condition. To further understand the activity of the plant, CME was partitioned successively with petroleum ether, chloroform, ethyl acetate and water, and the resulting fractions were evaluated similarly. Among the fractions, EAF showed the highest activity with 37% inhibition of AChE activity followed by AQF, CLF and PEF with 31%, 22% and 14% inhibition, respectively. Our results demonstrate that *A. marmelos* has a moderate AChE inhibitory activity.

Antioxidant activity

Reducing power assay

The reducing capacity of a compound is a significant indicator of its potential antioxidant activity because of its ability to break the free radical chain through donation of a hydrogen atom. The reducing capacity of the different extracts was evaluated by the method described by Oyaizu,[38] which is based on the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron. Figure 1 depicts the reducing power of CME, EAF, CLF, AQF, PEF and the reference standard AA. All the test extracts showed considerable reducing power; 100–600-mg/mL EAF, CLF, AQF, PEF and CME gave an absorbance within the 0.025–2.835 range. All the extracts increased the absorbance significantly, further demonstrating the antioxidant potential of *A. marmelos*. The reducing power of the different extracts and reference standard was in the following order: PEF<CME<CLF<AQF<EAF<AA.

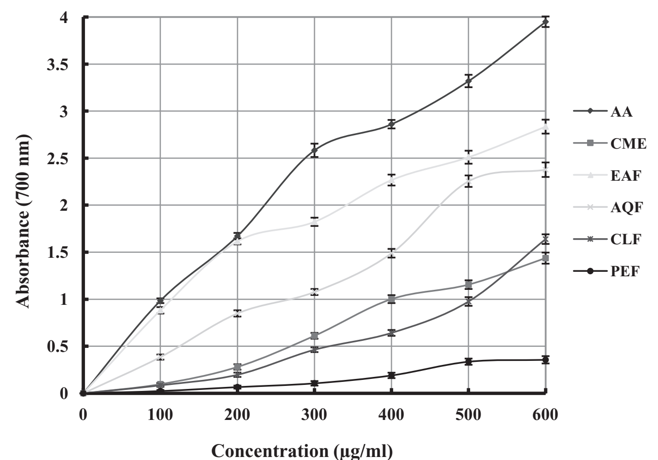

Figure 1 The reducing power of different *Aegle marmelos* extracts compared with the standard. Reducing power was measured by ferric reducing power assay. Results are mean ± SD ($n = 3$). AA, ascorbic acid; AQF, aqueous fraction; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; PEF, petroleum ether fraction.

DPPH radical scavenging activity

Radical scavenging activities are very important to prevent the deleterious role of free radicals in AD. The stable DPPH radical scavenging model is a widely used method to evaluate the free radical scavenging ability of various samples, including plant extracts, because of the relatively short time required for analysis. DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolourize in the presence of antioxidants that can be quantitatively measured from the change in absorbance; the percentage of scavenging activity is then calculated. The results of DPPH radical scavenging assays of CME, EAF, CLF, AQF, PEF and butylated hydroxytoluene are given in Figure 2. A significant decrease in the concentration of DPPH radical was observed due to the test extracts' free radical scavenging ability. The percentage of scavenging DPPH radicals at various concentrations of the extracts indicated that EAF has potential scavenging activity with a half maximal inhibitory concentration (IC_{50}) value of 3.84 ± 0.18 μg/mL, which is comparable to that of the reference standard butylated

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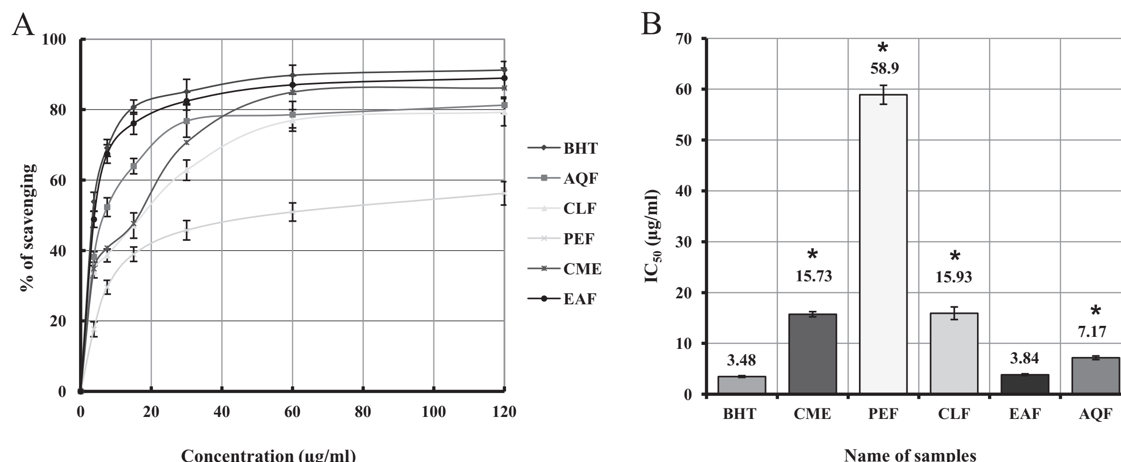

Figure 2 DPPH radical scavenging activity of different *Aegle marmelos* extracts compared with the standard. DPPH radical scavenging activity was measured spectrophotometrically using DPPH free radicals. (a) Percentage of DPPH radical scavenging by different concentrations of the extracts and reference standard BHT. Results represent mean ± SD ($n = 3$). (b) IC_{50} (mean ± SD) for DPPH radical scavenging activity of the extracts and BHT. *Values that significantly differed ($P < 0.05$) from the reference standard. AQF, aqueous fraction; BHT, butylated hydroxytoluene; CLF, chloroform fraction; CME, crude methanol extract; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EAF, ethyl acetate fraction; IC_{50}, half maximal inhibitory concentration; PEF, petroleum ether fraction.

hydroxytoluene ($IC_{50} = 3.48 \pm 0.17$ µg/mL). AQF, CME and CLF also showed good antiradical activity with IC_{50} values of 7.17 ± 0.36, 15.73 ± 0.51 and 15.93 ± 1.24 µg/mL, respectively. PEF had the lowest activity with an IC_{50} of 58.90 ± 1.86 µg/mL.

Hydroxyl radical scavenging activity

Several studies have indicated that Abeta induces apoptosis and neuronal cell death by producing ROS, which leads to the peroxidation of membrane lipids and oxidative stress.[15,19,20] Hydroxyl radicals are the major reactive oxygen species causing lipid oxidation and enormous biological damage.[43] To determine the extracts' hydroxyl radical scavenging capacity, the effect of each extract on hydroxyl radical generated in an Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction) was analyzed by evaluating the degree of deoxyribose degradation (Fig. 3). All the extracts scavenged the hydroxyl radicals generated in the reaction and prevented degradation of deoxyribose. The hydroxyl scavenging activity of all the extracts was significant. The results demonstrated that EAF and AQF are strong hydroxyl radical scavengers with respective IC_{50} values of 5.68 ± 0.41 and 6.36 ± 0.58 µg/mL, which appeared to be similar to that of the reference standard CA with IC_{50} of 6.50 ± 0.27 µg/mL. In contrast, CME, CLF and PEF showed similar activity with IC_{50} of 10.95 ± 0.56, 11.69 ± 0.69 and 13.25 ± 0.56 µg/mL, respectively.

Lipid peroxidation inhibition activity

Lipid peroxidation has been reported to be elevated in the brains of AD patients.[15,44,45] Reactive oxygen species produced by Abeta protein have numerous pathological effects including lipid peroxidation and cellular degeneration in AD. During lipid peroxidation, low molecular weight end products, generally malonaldehyde, are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of thiobarbituric acid to produce a pinkish red chromogen.[46] In the lipid peroxidation assay, the activity of *A. marmelos* extract against non-enzymatic lipid peroxidation in rat brain homogenate was evaluated (Table 2). The addition of Fe^{2+}-ascorbate to the brain homogenate caused an increase in lipid peroxidation. All the test extracts significantly inhibited brain lipid peroxidation in a concentration-dependent manner. The percentage of lipid peroxidation inhibition at the different concentrations of the extracts indicated that CME possessed the greatest potential inhibitory activity. This extract inhibited lipid peroxidation by 47% at a concentration of 100 µg/mL, while CA, used as the reference standard, decreased the lipid peroxidation by 59% under the same experimental condition. Among the different fractions, EAF and

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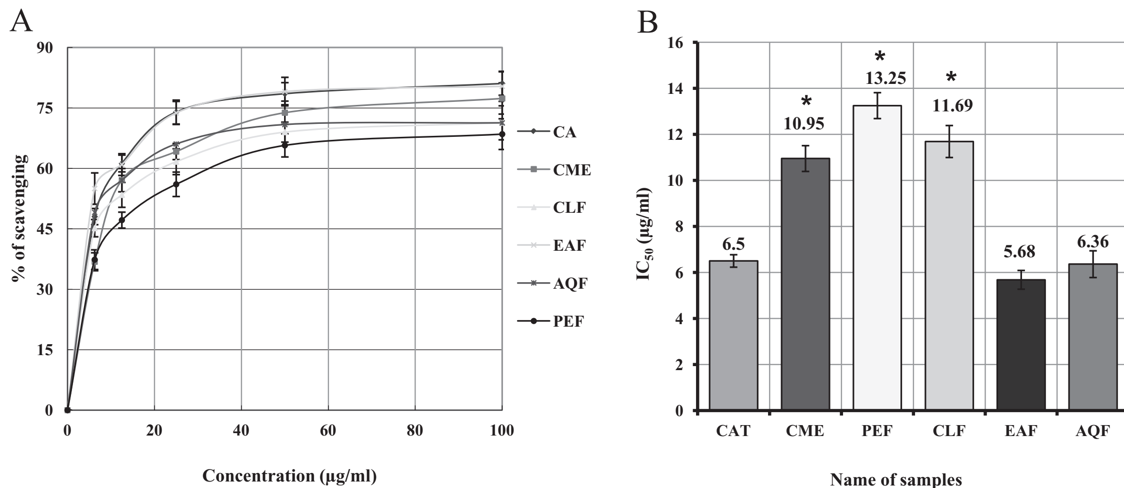

Figure 3 Hydroxyl radical scavenging activity of different *Aegle marmelos* extracts compared with the standard. Hydroxyl radical scavenging was measured by the Fenton reaction initiated deoxyribose degradation method. (a) Percentage of hydroxyl radical scavenging by different concentrations of the extracts and reference standard CA. Results represent mean ± SD ($n = 3$). (b) IC_{50} (mean ± SD) for hydroxyl radical scavenging activity of the extracts and CA. *Values that significantly differed ($P < 0.05$) from the reference standard. AQF, aqueous fraction; CA, (+) catechin; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; IC_{50}, half maximal inhibitory concentration; PEF, petroleum ether fraction.

Table 2 Inhibition of hydrogen peroxide-induced lipid peroxidation in rat brain homogenates by different extracts of *Aegle marmelos*

	Inhibition %)					
Concentration (µg/mL)	CME	PEF	CLF	EAF	AQF	Catechin
50	23.39 ± 1.63[b]	18.75 ± 1.40[a]	21.79 ± 1.99	25.77 ± 1.67[c]	31.28 ± 1.69[d]	47.34 ± 2.57[e]
100	47.81 ± 2.32[e]	24.50 ± 1.83[a]	30.17 ± 2.52[b]	37.61 ± 2.28[c]	40.29 ± 1.88[d]	59.85 ± 4.37[f]
200	53.95 ± 1.96[e]	25.77 ± 1.36[a]	30.67 ± 2.14[b]	39.55 ± 2.57[c]	42.19 ± 2.65[d]	65.54 ± 2.27[f]

AQF, aqueous fraction; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; PEF, petroleum ether fraction. [a–f]Means between columns differ significantly ($P < 0.05$) in each concentration.

AQF exhibited higher inhibitory activity that reduced lipid peroxidation by 38% and 40%, respectively, at the same concentration.

Phytochemical screening

The phytochemical screening of different *A. marmelos* extracts was done to ascertain the presence or absence of bioactive components; qualitative results are shown in the Table 3. The analyses revealed the presence of tannins, alkaloids, flavonoids, saponins, glycosides and steroids. Quantitative analysis of phenolics and flavonoids revealed that CLF had the highest total phenolic content (61.67-mg GAE/g dried extract), followed by EAF (58.79-mg GAE/g dried extract), PEF (40.70-mg GAE/g dried extract) and AQF (26.55-mg GAE/g dried extract) (Table 4). Similarly, the highest total flavonoid content was observed in CLF (410.4-mg GAE/g dried extract), followed by EAF (375.73-mg GAE/g dried extract), PEF (234-mg GAE/g dried extract) and AQF (204.35-mg GAE/g dried extract). Our results clearly demonstrated that EAF, which was found to possess the highest AChE

Table 3 Qualitative phytochemical screening of different extractives from *Aegle marmelos*

	CME	PEF	CLF	EAF	AQF
Tannins	+	–	–	–	+++
Flavonoids	+	+	+++	++	++
Phenolic compounds	+	+	++	++	+
Alkaloids	+	–	–	–	++
Saponins	+	–	–	–	+++
Steroids	+++	++	+	–	–
Glycosides	+	++	+	+	+++

AQF, aqueous fraction; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; PEF, petroleum ether fraction.

Table 4 Total phenolic and flavonoid contents of different extractives from *Aegle marmelos*

	Total phenolic content (mg GAE/g dried extract)	Total flavonoid content (mg GAE/g dried extract)
CME	46.73 ± 0.51^{c}	246.13 ± 3.60^{c}
PEF	40.70 ± 1.77^{b}	234.00 ± 3.38^{b}
CLF	61.67 ± 3.74^{e}	410.40 ± 5.77^{e}
EAF	58.79 ± 5.07^{d}	375.73 ± 4.01^{d}
AQF	26.55 ± 2.04^{a}	204.35 ± 6.27^{a}

AQF, aqueous fraction; CLF, chloroform fraction; CME, crude methanol extract; EAF, ethyl acetate fraction; GAE, gallic acid equivalent; PEF, petroleum ether fraction. [a–f]Means between columns differ significantly differ significantly ($P < 0.05$).

inhibitory activity and antioxidant property, contained a large amount of phenolics and flavonoids.

DISCUSSION

AD is a severe neurodegenerative disease and a leading cause of death among the elderly. Although a number of factors have been identified, oxidative stress and cholinergic dysfunction have been implicated as major contributing factors in the pathogenesis of AD.[1–5] Therefore, it has been suggested that the compounds, either plant or plant-derived molecules, that modulate cholinesterase activity and multiple components of the oxidative stress pathway would be an effective candidates for potential drugs that restrict the development of AD. *A. marmelos* has been reported to possess anti-diabetic, anti-hyperlipidaemic, gastroprotective, antidiarrhoeal, radioprotective, antimicrobial and antiproliferative properties.[28–34] Our findings indicate that *A. marmelos* also possesses AChE inhibitory properties and antioxidative activities.

It is now well accepted that inhibition of AChE activity can ameliorate the symptoms and improve the functioning of patients with AD.[10,11] The results of this *in vitro* study revealed that CME of *A. marmelos* inhibited rat brain AChE in a dose-dependent manner (Table 1). To further understand the activity of the plant, CME was partitioned successively with petroleum ether, chloroform, ethyl acetate and water, and the resulting fractions were evaluated similarly. The highest activity (37%) was found in EAF and was higher than the activity of CME (29%). Under the same experimental condition, donepezil, an anti-AD drug with potential AChE inhibitory activity, inhibited the activity by 97%, which is consistent with results published earlier.[47] However, our results demonstrated the moderate AChE inhibitory activity of *A. marmelos*.

The antioxidant potential of the different *A. marmelos* extracts was evaluated by several *in vitro* assays, including DPPH radical scavenging assay, hydroxyl radical scavenging assay and lipid peroxidation assay, as no single assay reflects the antioxidant property. As a preliminary study to estimate the antioxidant abilities of the test extracts, several assays that are not biologically relevant, such as reducing capacity and DPPH free radical scavenging assays, were performed. All the extracts showed radical scavenging activity and iron reducing ability in a concentration-dependent manner (Figs. 1,2). Among the different extracts, EAF showed the highest activity, and the activity of the other three fractions was in the following order: AQF>CLF>PEF.

Extensive evidence indicates that the ROS, which are formed during oxidative stress, induce cellular and molecular abnormalities in sporadic AD.[13–16] Hydroxyl radicals are the major ROS and are highly toxic; they greatly contribute to oxidative stress and are known contributors to neuronal damage in AD.[48] The results revealed that all the extracts possessed potential hydroxyl radical scavenging activity (Fig. 3). Among the extracts, EAF showed the highest activity with a IC_{50} of 5.68 μg/mL, which appeared to be more potent than that of the reference standard used in this study, CA with a IC_{50} of 6.5 μg/mL under the same condition.

Lipid peroxidation is the mechanism by which lipids are attacked by ROS to form a carbon radical that reacts with oxygen, resulting in a peroxyl radical and thus generating lipid peroxides.[15,44,45] Due to the high lipid content and unusually high concentration of polyunsaturated fatty acids that are particularly susceptible to oxidation, the brain is an important target of oxidative stress. The brains of individuals with AD have increased levels of lipid peroxidation products, such as 4-hydroxynonenal or 2-propenal, and enhanced lipid peroxidation has been detected in the cerebrospinal fluid and plasma of individuals with AD.[49] Lipid peroxidation products can be measured by using thiobarbituric acid.[46] The results of an *in vitro* assay revealed that, induced by hydrogen peroxide in a concentration-dependent manner, CME of *A. marmelos* inhibited peroxidation of brain lipid (Table 2). The activity of CME was found to be higher than the other four fractions, indicating the presence of

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inhibitors of lipid peroxidation acting additively or synergistically in the mother CME.

The AChE inhibitory property and antioxidant activity of *A. marmelos* were supported by the presence of endogeneous polyphenols and flavonoids, tannin, alkaloids, and steroids (Table 3). Polyphenols are the most abundant antioxidants in the plant kingdom, and it is claimed they have neuroprotective effect.[50] The antioxidant activity of the polyphenolic compound is believed to result from their redox properties, which play an important role in adsorbing and neutralizing free radicals. Flavonoids are the most ubiquitous groups of plant secondary metabolites and have good antioxidant potential.[51] The mechanism of action of flavonoids is through the scavenging of free radicals. Numerous ployphenolic and flavonoid compounds have been isolated from plants that reduce oxidative stress and inhibit AChE.[52–56] Our results indicate that EAF derviced from *A. marmelos* is a significant source of phenolics and flavonoids, which may contribute to inhibition of AChE and antioxidant activity (Table 4).

Conclusion

The results suggest that EAF derived from *A. marmelos* effectively inhibits AChE activity and multiple components of the oxidative stress pathway that can contribute to Alzheimer's pathology. As a result, EAF has the potential to be an effective and safe treatment for AD. The validity of this plant as folkloric medicine is now being evaluated critically in cell-free systems.

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