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Identification of acetylcholine esterase inhibitors from *Morus alba* L. leaves

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ABSTRACT

The entire plant extract of Morus alba L. has been widely used in traditional medicine for treating various disorders such as diabetes and also as health tonic. But there is no report available so far regarding the presence of acetylcholine esterase inhibitors in this plant. The aim of the present study was to test the M. alba leaf extract for acetylcholine esterase inhibitory activity. The 50% methanolic extract of M. alba was tested for its in vitro acetylcholine esterase inhibitory activity using modified Ellmann's method. The crude methanolic showed acetylcholine esterase inhibitory activity in a concentration dependent manner and around 10 µg of the extract was required for 50% inhibition of the activity. The major compounds in the extract were identified using RP-HPLC analysis as vanillic acid, myricetin, luteolin and kaempferol. Out of this four major compounds, myricetin, luteolin and kaempferol showed significant acetylcholine esterase inhibitory activity when tested individually. So I conclude that the M.alba leaf extract possess significant acetylcholine esterase inhibitory activity and the compounds responsible for this effect were identified as myricetin, luteolin and kaempfeol. This is the first study on the identification of acetylcholine esterase inhibitors from M.alba.

Keywords Mulberry, Myricetin, Luteolin, Kaempferol

INTRODUCTION

Alzheimer's disease is a progressive, neurodegenerative disease affecting especially elderly population resulting in impaired memory and behavior. Reversible inhibitors of choline esterase are widely used for treating this disease. These compounds will help in increasing the levels of acetylcholine level in the brain [1]. Nature is a rich source of compounds with unique and complex structure that cannot be easily synthesized by chemical methods. Identification of highly efficacious acetylcholine esterase inhibitors (AchEIs) from natural sources has now become important because the use synthetic ones have serious side effects such as gastrointestinal disturbances. The most potent reversible inhibitor of acetylcholine esterase (AchE) isolated from natural source is Huperzin A from a Chinese traditional medicine *Huperzia serrata* [2].

Morus alba L. (Mulberry) is widely being used in traditional medicine for treating various diseases. Many flavones were isolated from the root bark as active principles [3]. *M.alba* leaves contain rutin, quercetin and apigenin as bioactive constituents [4]. There is no report available so far regarding the presence of AchEI in *M.alba*. Recently another species of mulberry was screened for inhibitors of acetylcholine esterase and butyrylcholine esterase and have identified nine new flavones [5]. To the best of our knowledge this is the first study revealing the information regarding the identification of some important compounds as AchEIs from *M.alba*.

MATERIALS AND METHODS

Reagents

All the chemicals and reagents used were of high quality analytical grade reagents. 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylcholine esterase, acetylcholine iodide, and all the standards for HPLC analysis were purchased from from Sigma, USA. Methanol and acetic acid were purchased from Sisco Research Laboratory, India.

Preparation of the extract

M. alba L. leaves collected during March-April season were used for the study. About 20 g of leaves were washed, air dried and macerated with 100 ml 50% methanol in water for 48 hours at 4^0C. The extract was filtered through double layered cheese cloth and centrifuged 10,000 rpm for 10 minutes. The supernatant was collected, the methanol evaporated using rotavapor and then lyophilized. The dried extract was stored at 4^0C and was used for the *in vitro* analysis.

Inhibition of acetylcholinesterase activity- In vitro assay

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method as modified by Eldeen *et al*. [6]. Into a 96-well plate was added 25 µl of 15 mM acetylcholine iodide (ATCI) in water, 125 µl of 3 mM DTNB in Buffer C (50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M MgCl2.6H2O), 50 µl of Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin) and 25 µl of crude methanolic extract of *M.alba* prepared dissolved in 100% methanol at different concentrations (0, 2, 5, 10, 20 µg). Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 1 minute in microplate reader (FLUOstar optima, BMG Labtech, Germay) at 405 nm. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

$$\% \text{ Inhibition} = 1-(A_{sample} / A_{control}) \times 100$$

where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank [methanol in Buffer A (50 mM Tris–HCl, pH 8)].

HPLC analysis of the extract

RP-HPLC analysis of the crude methanolic extract was performed in a Waters 1525 HPLC system equipped with a binary pump system, UV visible detector (Waters model 2487) and syringe loading sample injector with loop size of 20 µl and an auto sample injector. The separation was done using a C-18 column (150x4.6nm, I.D. 5 µM) protected by a guard column with column oven temperature maintained at 25 ºC. The elution was carried out with gradient solvent systems with a flow rate of 1 ml/ min at ambient temperature (25-28°C). The mobile phase was consisted of 0.1 %v/v acetic acid (solvent A) and HPLC grade methanol (solvent B). The mobile phase was prepared and filtered through a 0.45 µm and sonicated before use. Total running time was 20 min and the gradient programme was as follows: 90 % A and 10% B for 0- 5 min, 70 % A to 30 % B for 3 min, 55 % A to 45 % B for 3 min, 30% A to 70%B for 2 min, 20% A to 80% B for 2 min, and 100% B for 5 min. The sample injection volume was 20 µl and the wavelength of the UV-VIS detector was set at 255 nm. The retention time of all the standards were also determined using the same protocol.

Statistical analysis

All the experiments were performed in triplicate and the values are expressed as mean±SD.

RESULTS

In vitro inhibition of acetylcholine esterase activity by M.alba extract

Different concentrations of methanolic extract of *M.alba* (2, 5, 10 and 20 µg) were tested for the inhibitory effect of acetylcholine esterase activity. The results are given in Fig. 1. The extract showed a concentration dependent inhibition on AchE activity and around 10 µg of extract was needed for the 50% inhibition of the activity.

HPLC analysis for the identification of active components in the extract

The crude methanolic extract which showed AchE inhibitory activity was subjected to HPLC analysis for the identification of its active principles. By comparing the retention of various peaks obtained with the retention time

of various standards, the active principles were identified. The extract contains ascorbic acid (2%), gallic acid (1.2%), chlorogenic acid (2.8%) as the minor components and vanillic acid (22%), myricetin (15%), luteolin (32%) and kaempferol (15%) as the minor components. The results are given in Fig.2 A.

AchE inhibitory activity of separated major components of M.alba

The peak fractions of the major components (vanillic acid, myricetin, luteolin and kaempferol) were collected evaporated off the solvent and 5 µg of each compound were analyzed for AchE inhibitory activity. The results are shown in Fig. 2B. Vanillic acid did not show any AchE inhibition, but all other compounds showed inhibition with varying percentages. Myricetin showed 68%, luteolin showed 76% and kaempferol showed 52% inhibition of AchE activity.

DISCUSSION

Choline esterase inhibitors have been reported to be effective in treating memory impairment and other cognitive dysfunctions associated with Alzheimer's disease. Plant derived AchE inhibitors, especially flavonoids could be appropriate in treating this condition because of less side effects and many such compounds have been reported earlier [7, 8]. For the first time, we are reporting here the acetylcholine esterase inhibitory activity of *M.alba* leaves.

Figure 1 Percentage inhibitory activity of the methanolic extract of *M.alba* on AchE inhibition: Different concentrations of crude methanolic extract (2, 5, 10 and 20 µg) were tested for the AchE inhibition and the percentage inhibition was calculated. Around 10 µg of extract needed for 50% inhibition. Values given are average of three independent experiments± standard deviation.

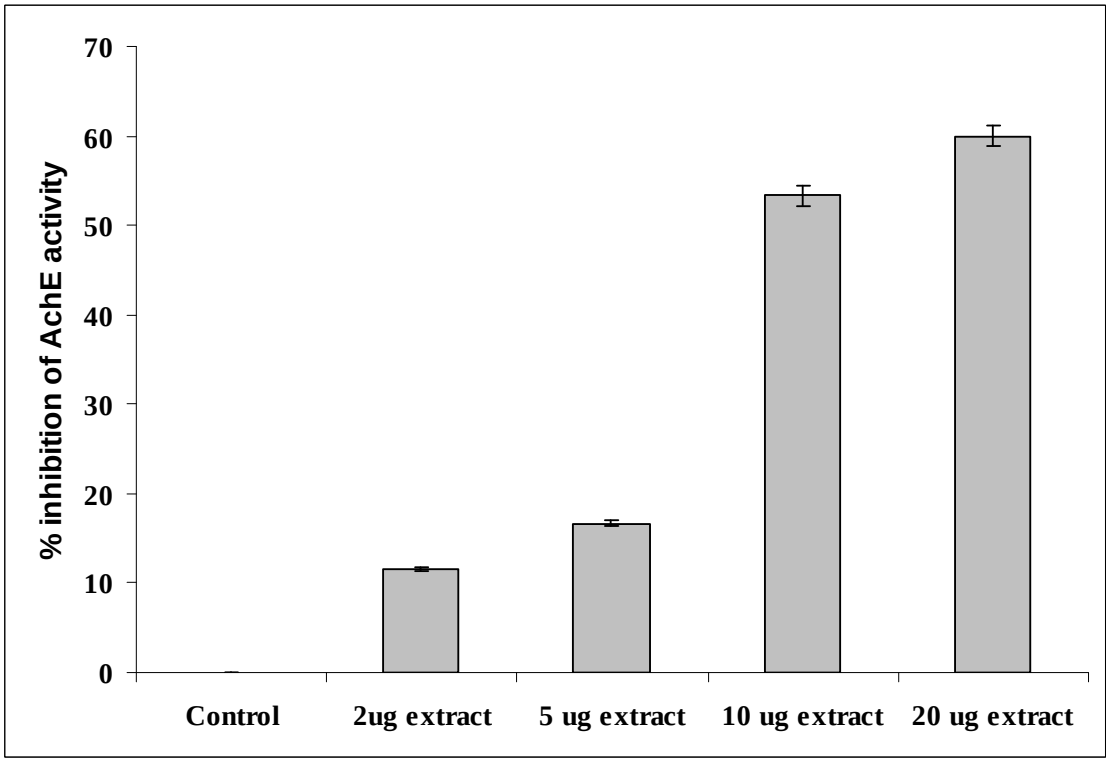
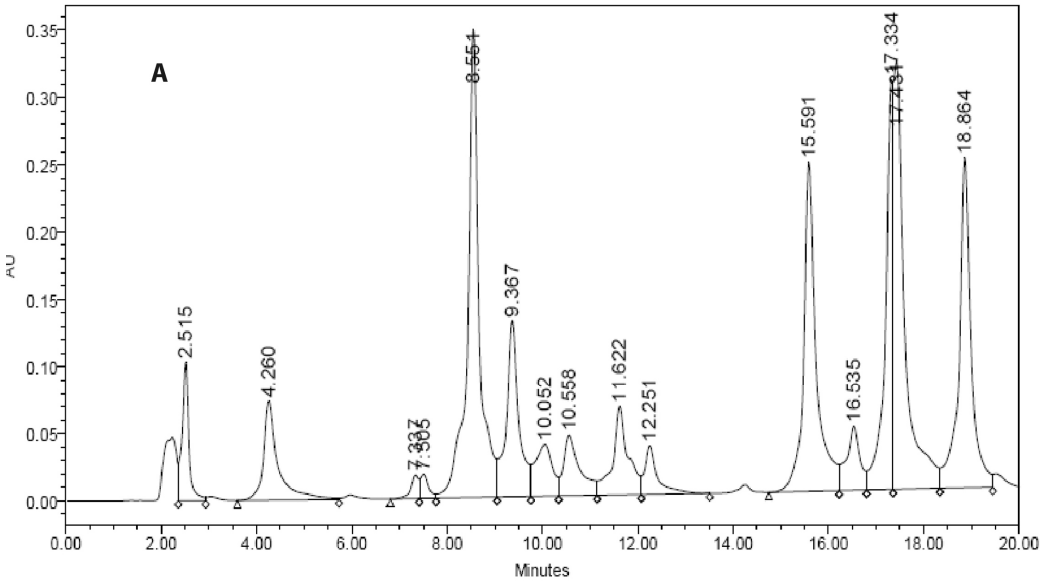
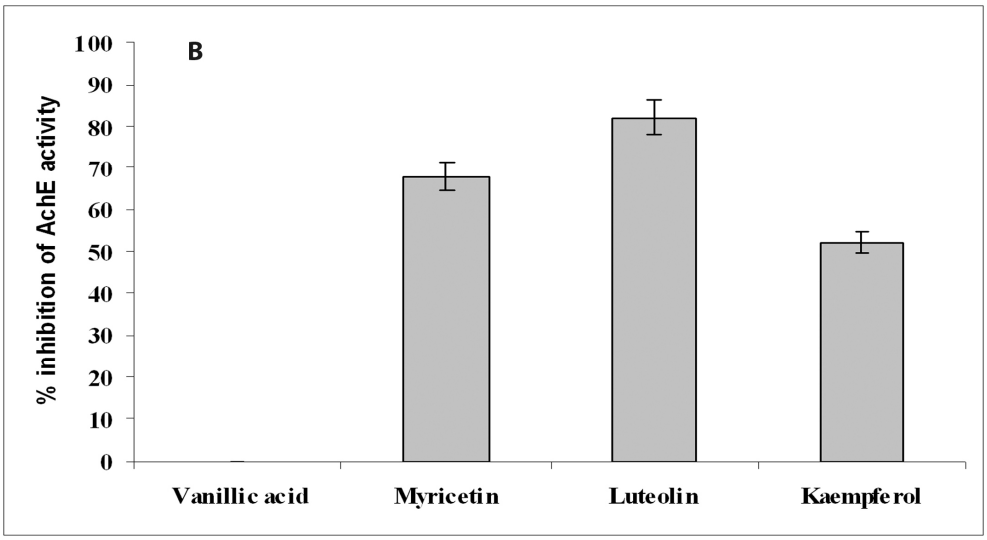


Figure 2 HPLC profile of crude methanolic extract of *M.alba*. Major and minor peaks were identified by comparing retention time (Rt) of each peaks with Rt of various standards (A). Percentage inhibition of AchE inhibitory activity of 5µg of each of the major compounds (vanillic acid, myricetin, luteolin and kaempferol) purified from extract (B). Luteolin showed maximum AchE inhibitory activity. Values given are average of three independent experiments± standard deviation.



Peak	Retention time (Rt)-min	Compound identified
Peak 1	2.515	Ascorbic acid (Rt-2.514)
Peak 2	4.260	Gallic acid (Rt-4.189)
Peak 3	8.551	Vanillic acid (Rt-8.354)
Peak 4	9.367	Chlorogenic acid (Rt-9.909)
Peak 5	15.591	Myricetin (Rt-15.346)
Peak 6	17.334	Luteolin (Rt-17.332)
Peak 7	18.864	Kaempferol (Rt-18.725)



The leaves of *M.alba* is one of the best known medicinal herbs used in the treatment of diabetes and the beverages containing this leaves promote good health [9]. The bioactive components of this leaves were investigated before and it contains many flavones, steroids, tripterpenes and many nitrogen containing sugars. Out of these N-containing sugars, deoxynojirimycin and fagomine have most potent hypoglycemic effects. Besides this, the plants also possess antiproliferative activity. Albanol A derived from the root bark induces apoptotic cell death in leukemic cancer cells [10]. The leaves also possess immunomodulatory [11] and anti-inflammatory effects [12]. Eventhough *M.alba* is used in the treatment of many diseases, there are no reports available till now on the acetylcholine esterase inhibitory activity of this plant. In the present study we have reported fro the first time, three major compounds (myricetin, luteolin and kaempferol) from this plant are able to inhibit the AchE activity. These results clearly indicating the wide possibility of this plant to be used in the treatment of Alzheimer's disease.

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