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Studies of acetylcholinesterase inhibition by herbal extracts with vitamin B_6 *in vitro* and *in vivo*

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SUMMARY

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes acetylcholine to choline and acetate. In the previous reports the level of acetylcholine (ACh) was decreased in the brain of Alzheimer patients, which has led us to focus on the research to develop a treatment inhibiting AChE. Thus we have discussed here the effects of herbal extracts (HAN and Ginkgo) with vitamin B_6 on AChE activities *in vitro* and *in vivo*. As concentrations of herbal extracts and vitamin B_6 increased, the value of reaction rate of AChE was decreased for the substrate acetylthiocholine (ATCh). When the herbal extracts and vitamin B_6 were mixed, the value of reaction rate of AChE was also decreased *in vitro* state. And they act as reversible noncompetitive type inhibitors, as shown in Lineweaver-Burk plot. For an *in vivo* study we administrated orally the herbal extracts and vitamin B_6 to rats for 10 consecutive days. The activity of AChE in brain tissues of rats was decreased. As a result, the AChE activity was inhibited by the herbal extracts and vitamin B_6, and they could be good drug candidates for curing Alzheimers disease.

Key words: Acetylcholinesterase inhibitor, Herbal extracts, HAN, Ginkgo, Vitamin B_6, Rat brain, Alzheimer's disease

INTRODUCTION

Acetylcholinesterase (E.C. 3.1.1.7, AChE) is an enzyme that hydrolyzes acetylcholine to choline and acetate (Quinn, 1987; Bartus *et al*., 1982). AChE inhibitors have been used as components of nerve gases but also have significant medical applications in the treatment of disorders such as glaucoma and myasthenia gravis as well as in terminating the effects of neuromuscular blocking agents such as atropine. For the physiological importance of AChE in the neurotransmission, the AChE inhibitors are used as chemical warfare agents, insecticides, and anti-Alzheimer's disease drugs. Tacrine (THA, Cognex®) and E2020 (Donepezil hydrochloride, Aricept®) are reversible inhibitors used as anti-Alzheimer's disease drugs (Summers *et al*., 1981; Summers *et al*., 1986; Rogers *et al*., 1996; Roger and Friedhoff, 1996). They improve the cognitive functions, but they don't stop the progression of the disease. Also, tacrine exhibits some side effects in the liver, and Donepezil shows few harmful side effects (Ames *et al*., 1990; O'Brien *et al*., 1991; Delagarza, 1998). Therefore, it seems necessary to continue to seek for new AChE inhibitors with markedly reduced side-effects and greater therapeutic windows. *Ginkgo biloba* can improve brain function, including memory. Ginkgo also has a capacity to increase the oxygen content of blood. It has been shown that memory is enhanced by oxygen-rich blood. All neural functions in the brain are achieved by neural transmission. *Ginkgo* increases

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both the amount of neural transmission and the number of receptor sites for neural transmission. Huperzine A which is a natural alkaloid isolated from *Huperzia serratax* is a very potent inhibitor of AChE (Ashni *et al*., 1992; Tang, 1996; Tie Wang *et al*., 1998) Vitamin B_6 is a water-soluble vitamin that helps the body to synthesize proteins which are then used for body cells to proliferate, that is also used to convert tryptophan (an amino acid) into niacin and serotonin (a brain chemical), that activates body functions to produce other life supporting biochemicals such as insulin, hemoglobin and antibodies to fight infections. In this respect, herbal extracts and vitamin B_6 have a potential for an anti-Alzheimers disease treatment and are beneficial because of low cytotoxicity. But there are currently no exact certifications for their efficacies and the mechanism of their action on treating Alzheimers disease except clinical results. In this paper, we described inhibitory activities of new herbal extracts (HAN), *Ginkgo biloba* and vitamin B_6 on AChE *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Animals

Three-week-old female Sprague-Dawley rats were purchased from Samyook Co., Korea. All rats were fed with commercial animal pellets (Samyook Co., Korea) and tap water. Animal room maintained at a temperature of 23±3°C, a relative humidity of 50±10%, and illumination cycle of 12 hr light and 12 hr dark (light during 07:00~19:00). Rats were used from the age of 21 weeks (216~359 g).

Preparation of extract powder

A mixture consisting of 50 g of ginseng, 15 g of *Arisaematis rhizoma*, 15 g of *Gastrodiae rhizoma*, 10 g of *Acorus graminecus*, 10 g of *Ostericum koreanum*, 10 g of *Bambusae caulis*. In Taeniam, 10 g of *Bombycis corpus*, 10 g of *Ponciri fructus*, 10 g of Hoelen, 10 g of Pinelliae t*uber*, 6 g of *Aurantii nobilis pericarpium* and 6 g of *Glycyrrhizae radix* was placed in 1 L of water, heated slowly and refluxed at 100°C for 6~8 hours. Then, the liquid-phase was filtered with a Whatman 541 (110 mmΦ) filter paper. The filtrate was completely dried to 12.2 g of brown powder (HAN) using a freeze vapor drier (F. D. 8510, Ilshin Lb Co., Korea). 4 g of *Ginkgo biloba L.* was diluted with 10 ml of water and filtered with a filter paper (Whatman 541: 110 mmΦ). The filtrate was dried using a freeze vapor drier. 400 mg of vitamin B_6 was dissolved in 10 ml of water and prepared fresh daily.

Assay for the *in vitro* inhibitory activity of herbal extracts to acetylcholinesterase

Various concentrations of freeze dried extracts were used to determine whether herbal extracts inhibit AChE (Type V-S; from Electric Eel., Sigma Co., USA) or not. Concentration of each extract sample in a cuvette is 1.00, 2.00 and 4.00 mg / ml for HAN; 0.1, 0.2 and 0.4 mg / ml for *Ginkgo biloba L.*; and 0.01, 0.02 and 0.04 mg / ml for vitamin B_6.

The 990 µl of reaction mixture containing 0.1 M PBS (pH 7.3), 5 mM acetylthiocholine (ATCh), 60 µl of 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 10 µl of herbal extracts were incubated at 25.0°C for 3 minutes, and then 2 unit / 10 µl of AChE was added to the mixture. ATCh is hydrolyzed to acetate and thiocholine that reacts with DTNB to produce 5'-thio-2-nitrobenzoate, and the light absorbance measured at 412 nm (Ellman *et al*., 1961). The light absorbance was measured with various concentrations of ATCh and extract powder at different incubation times. Analyses were run at least in triplicates and the enzyme reaction rate on all substrates at the intervals of incubation was calculated by Michaelis-Menten equation. Five different concentrations of each compound were used, in order to obtain inhibition of AChE activity comprised between 20 and 80%. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100\text{-}V_i / V_0 \times 100$ where V_i is the rate calculated in the presence of inhibitor and V_0 is the enzyme activity. Inhibition curves were obtained for each compound by plotting the percent inhibition versus the logarithm of inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve and the IC_{50} values extrapolated.

Assay for the activity of acetylcholinesterase in the crude rat brain homogenate

To evaluate the inhibitory effect of herbal extracts *in vivo*, rats were divided into three groups (control,

HAN treatment and extract mixture treatment groups) consisting of 10 animals each prior to the start of administration. HAN treatment group was administrated with 0.8 g/kg HAN extracts once a day for 10 consecutive days, and control animals with 3 ml saline. The extract mixture treatment group was administrated with 0.8 g/kg HAN extracts, 0.08 g/kg *Ginkgo biloba L.* and 0.008 g/kg vitamin B_6 once a day for 10 consecutive days. After 10 days, rats were anesthetized lightly with CO_2 and sacrificed by vertebral fracture. Brains were removed rapidly, frozen in liquid nitrogen and stored at -70°C until assayed. Kinetics of AChE activity in brain tissues was measured within two hours.

To prepare crude AChE extract 1.7 g of brain sample was added with 7.0 ml of 0.1 M PBS (pH 7.3) containing 0.2% Triton X-100 and homogenized (Ultra-Turrax T25). The homogenate was centrifuged (ROTINA 48R, Hettich Co., Germany) at 4.0°C, 1000 rpm for 10 min. The pellet was discarded and the supernatants was filtered through a 0.45 μm membrane micro filter (CAMEO 25ES). The activity of AChE in brain samples was analyzed rapidly by Ellmans coupled assay (Ellman *et al*., 1961). In brief, the reaction mixture containing 840 μl of 0.1 M PBS (pH 7.3), 80 μl of 5.0 mM ATCh and 60 μl of 5.0 mM DTNB was incubated at 25.0°C for 3 minutes, and then each 10 μl of crude brain homogenate extracted from the rats of treatment group was added to the reaction mixture for the assessment of AChE activity. Analyses were run at least in triplicates. AChE activity was measured using a UV-visible spectrophotometer at 412 nm. The enzymatic reaction rate on all substrates at the intervals of incubation time was calculated by Michaelis-Menten equation.

Statistical analysis

All values, expressed as the mean ±S.D., were statistically analyzed through analysis of students t-test. The p value less than 0.05 was considered as significant.

RESULTS

Clinical findings

There was no dead animal in all the groups throughout the experimental period. No distinguishable clinical sign was observed in the treatment group comparing to the control group. There was no statistically significant change in the body weight, and in food and water consumptions between the treatment and the control groups.

Effects of herbal extracts on acetylcholinesterse activity

The reaction rate of AChE at various concentrations of herbal extracts (HAN and Ginkgo biboba) and vitamin B_6 *in vitro* is summarized in Fig. 1. There are significant decreases for initial rate values at various concentrations of herbal extracts and vitamin B_6 compared to control.

The kinetic parameters for control and inhibition reactions were calculated by the integrated Michaelis-Menten equation as shown in Table 1. As the extract concentration increased, V_{max} and V_{max}/K_m were decreased, and K_m was constant. The IC_{50} values were reported in Table 2. The IC_{50} by HAN, *Ginkgo biloba L.* and vitamin B_6 for AChE was shown as 3.263, 0.364, and 0.121 mg/ml, respectively. These results suggest that vitamin B_6 has highest inhibition effect for AChE.

The Lineweaver-Burk plot of AChE is shown in Fig. 2. The lines have different intercepts on the 1/[S] axis, showing a constant in K_m from these plots. However all lines show an increment of the slope and of the intercept on the 1/velocity, demonstrating that herbal extracts is an effective inhibitor at both high and low substrate concentrations.

The activities of AChE in rat brain are summarized in Table 3. The initial reaction rates of AChE for HAN treatment and for extract mixture treatment groups were significantly decreased by 21.7% and 29.5%, to those of the control group relatively.

DISCUSSION

Acetylcholine (ACh), which was identified in the central nervous system, plays a significant role as a neurotransmitter of cholinergic neurons. ACh has received a renewed attention associated with Alzheimer's disease (AD), which is characterized by a decrease in cholinergic neurons. There are multiple neurotransmitter changes in the brain in Alzheimer's disease. ACh levels are considerably reduced and the degree of reduction

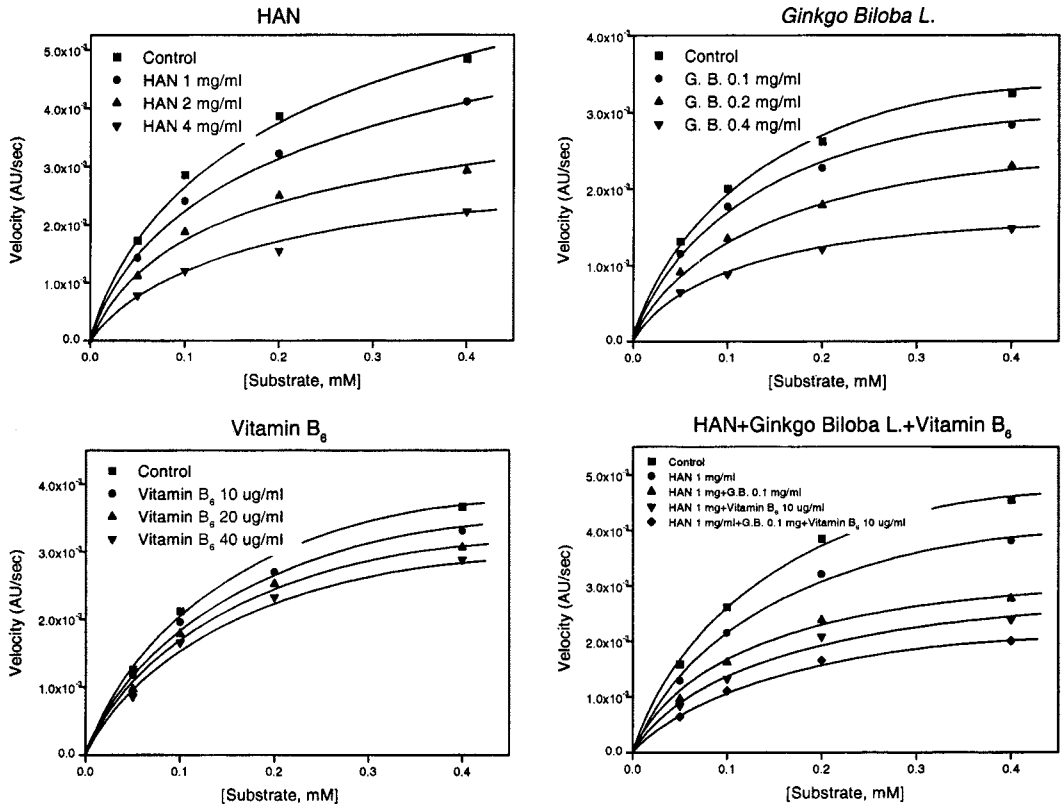

Fig. 1. The inhibition of acetylcholinesterase by various concentration of herbal extracts and vitamin B_6 *in vitro* experiments: This plot shows the reaction velocity versus to acetylthiocholine concentration at different concentrations of Inhibitors. The values are the mean for triplicate experiments (standard deviation<5%).

Table 1. Kinetic parameters of acetylcholinesterase reaction with a substrate acetylthiocholine and different concentrations of inhibitors

Compounds	[I] (mg/ml)	K_m (A)	$V_{max}\times10^3$ (A/s)	V_{max}/K_m (s^{-1})
HAN	0	0.1307±0.0082	6.4252±0.1616	0.0492±0.0197
	1.00	0.1360±0.0115	5.4896±0.1881*	0.0404±0.0164
	2.00	0.1064±0.0137	3.7603±0.1788**	0.0353±0.0130
	4.00	0.1616±0.0428	3.0385±0.3491**	0.0188±0.0082
Ginkgo biloba L.	0	0.1060±0.0057	4.0840±0.0808	0.0385±0.0142
	0.1	0.1039±0.0080	3.5388±0.0999*	0.0341±0.0125
	0.2	0.1176±0.0131	2.9332±0.1257**	0.0249±0.0096
	0.4	0.1003±0.0110	1.8325±0.0725***	0.0182±0.0066
Vitamin B_6	0	0.1385±0.0082	4.9795±0.1654	0.0360±0.0202
	0.01	0.1289±0.0115	4.4002±0.1114*	0.0331±0.0097
	0.02	0.1469±0.0137	4.2614±0.3002*	0.0290±0.0219
	0.04	0.1569±0.0428	4.0626±0.3128*	0.0259±0.0073
Extract Mixture	0	0.1354±0.0193	6.1948±0.3576	0.0458±0.0185
	HAN 1	0.1430±0.0214	5.2676±0.3260*	0.0368±0.0152
	HAN 1+G.B. 0.1	0.1309±0.0225	3.7520±0.2585**	0.0287±0.0115
	HAN 1+V_{B6} 0.01	0.1391±0.0308	3.2974±0.2996**	0.0237±0.0097
	HAN 1+G.B. 0.1+V_{B6} 0.01	0.1540±0.0218	2.8210±0.1701**	0.0183±0.0078

The data show the mean±standard deviations for triplicate experiments, *p<0.0005, **p<0.0001, ***p<1.00E-5: significantly different from the control group. G.B.: *Ginkgo biloba L.*, V_{B6}: Vitamin B_6.

Table 2. AChE inhibitory activity (IC_{50}) of Activities of HAN, *Ginkgo biloba L.* and vitamin B_6

Compounds	IC_{50} (mg / ml)
HAN	3.263±0.338
Ginkgo biloba L.	0.364±0.016
Vitamin B_6	0.121±0.008

The data show the mean±standard deviations for triplicate experiments.

correlates with the degree of cognitive impairment. Acetylcholinesterase (AChE) inhibitors are the first class of drugs shown efficacies in the treatment of Alzheimer's disease. They act by inhibiting AChE at cholinergic synapses throughout the nervous system, thereby increasing the availability of ACh within the brain. The first AChE inhibitor used in Alzheimer's disease, tetrahydroaminoacridine (THA or tacrine) is hepatotoxic and is not currently registered. Therefore, it is not considered as a further use. Rivastigmine (ENA-713) is a pseudo-reversible non-competitive acetylcholinesterase inhibitor with preferential selectivity for the hippocampus and cortex (Corina *et al.*, 1999). Donepezil (E2020) is a reversible, non-competitive acetylcholinesterase inhibitor with an elimination half-life of about 70 hours. Most of these are hampered to use by its own toxicity. If there is a drug that has equal efficacies as its predecessors or better without a harmful toxicity, it would be a good drug candidate. As such a drug, herbal extracts are drawing an attention in the light of showing less side effects with efficacies.

We have conducted studies using rats and *in vitro* analyses to determine if herbal extracts (HAN and *Ginkgo biloba*) and vitamin B_6 could be a medicine without side effects curing AD based on the hypothesis of cholinergic neuron. As the concentration of herbal extracts and vitamin B_6 increased in the reaction mixture, the initial rate value of AChE was decreased, which indicated that they inhibited AChE activity as in Fig. 1 and Table 1. These results proposed that herbal extracts and vitamin B_6 are

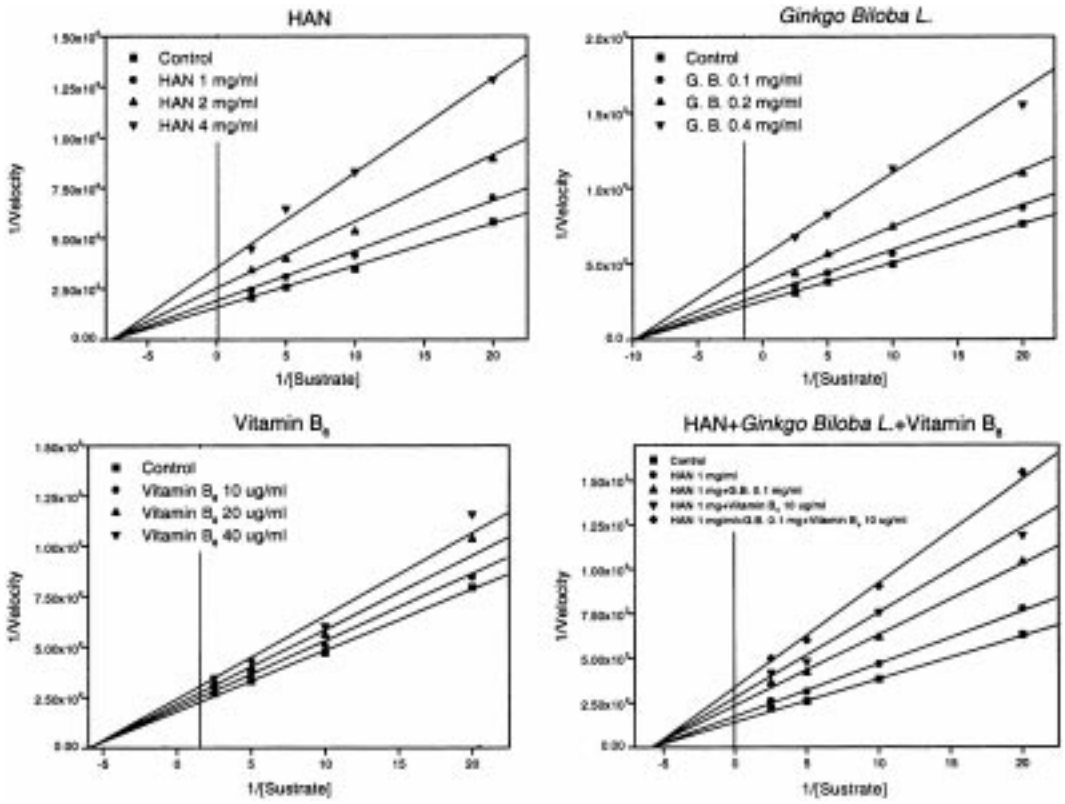

Fig. 2. The Lineweaver-Burk plot of acetylcholinesterase at different concentrations of herbal extracts and vitamin B_6. This plot shows 1 / reaction velocity versus 1 / [acetylthiocholine] in the presence of Herbal extracts and vitamin B_6. The values are the mean for triplicate experiments (standard deviation<5%).

Table 3. Activities of AChE in the rat brain homogenate

Treatment groups	AChE initial rate (AU / s)	Decrement %	N
Control	4.059±0.291 E-3	0	10
HAN	3.175±0.183 E-3*	21.7	10
Extract Mixture	2.314±0.202 E-3**	29.5	10

N: Number of rats used in the group. The data show the mean±standard deviations, *p<0.005, ** p<0.001: significantly different from the control group.

not competing with the substrate at higher substrate concentrations and inhibits equally well at low (V_{max}/K_m is decreased) and high concentrations of the substrate (V_{max} is decreased). Especially, at a higher concentration herbal extracts allows the substrate to bind, but reduces its affinity, and K_m resulted to constant, indicating that herbal extracts and vitamin B_6 act as non-competitive type inhibitors for AChE as shown in Fig. 2. The components of HAN have not known to contain any AChE inhibitors previously. These results suggest that HAN contains new AChE inhibitors, that various molecules in herbs had combined to form new molecules. It is known that the active site of AChE forms the narrow and long cleft (Sussman JL *et al*., 1991; Bourne Y *et al*., 1995). Therefore, these inhibitors are probably small and long straight molecules so that they can reach the active site of AChE. If they are very large molecules in size, binding of them to AChE may distort atom structures of the active site and cause the conformational change of AChE molecule, that resulted in inactivating the hydrolysis of ATCh in the experiment. Different inhibition mechanisms and inhibition constants (K_i) of various molecules in herbal extracts are now investigating. Recently, it was suggested that hydrophobic environments are close to the peripheral binding site of the AChE (Sugimoto *et al*., 1995). The six-membered ring of vitamin B_6 can stack against the aromatic amino acids of the peripheral binding site by a classical π-π interaction. Futhermore, vitamin B_6 can make a cation-π interaction with the peripheral binding site of AChE, because it has the tautomeric form with a positively charged nitrogen. This implicates that the substrate can't reach on an active site located at the bottom of gorge, and vitamin B_6 acts as a good inhibitor for AChE. We also found that the rate of decrease of V_{max} is much greater when 0.1 mg / ml of *Ginkgo biloba* or 0.01 mg / ml of vitamin B_6 was added to HAN reaction mixture than when a single compound of *Ginkgo biloba* or vitamin B_6) was added to the inhibition reaction (Table 1). This result can propose that the components of HAN inhibiting AChE may distort atom structures of the peripheral binding site, and these atom structures can let *Ginkgo biloba* and vitamin B_6 interact strongly with the peripheral site of AChE. This explanation is supported by the possibility that vitamin B_6 can stack structurally against the peripheral binding site by a classical π-π or cation-π interaction.

The AChE inhibition data expressed as IC_{50} values was shown in Table 2. HAN (3.263±0.338 mg / ml), *Ginkgo biloba L.* (0.364±0.016 mg / ml) and vitamin B_6 (0.121±0.008 mg / ml) were less effective than tacrine (49.6±1.98 ng / ml) (Piero *et al*., 1997). Tacrine is a purified single molecule synthesized chemically, and highly active. On the other hand HAN and *Ginkgo biloba L.* were herbal extracts composed with a number of compounds naturally found, and contain very low concentration of components inhibiting AChE. These natural compounds would be highly active as much as tacrine or more if purified compounds are used.

After oral administration of the herbal extracts for 10 consecutive days, there were no significant changes in the absolute brain weight of the control and the treated groups. The initial rates of AChE for the treatment group were significantly decreased relatively to the control group (Table 3).

These data strongly support that herbal extracts (HAN and *Ginkgo biloba L.*) and vitamin B_6 are very effective in treating AD, and equally effective to compare with commercially available drugs such as tacrine. In addition, considering the toxicity problems of tacrine and Donepezil, these herbal extracts and vitamin B_6 could be very valuable

medicine with little side effect to treat AD. More herbal extracts are now investigating in our laboratory, and which are appeared effective and promising in clinical study.

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