Review

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A systematic review on anti-Alzheimer's disease activity of prescription Kangen-karyu

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SUMMARY Traditional Chinese and Japanese medicines have become prime sources of drug discovery and there is a pressing need to investigate the effectiveness of these traditional medicines for modern drug discovery. Recently, among various traditional formulations, studies on Kangen-karyu (Guan-Yuan-Ke-Li), a mixture of six medicinal herbs (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma), have been growing to assess its neuroprotective role. This prompted us to undertake a thorough review of various targets of Kangen-karyu regarding its effectiveness against Alzheimer's disease, particularly focusing on cholinesterases, beta-site amyloid precursor protein cleaving enzyme 1, and glycogen synthase kinase 3β. This review provides new insights into Kangen-karyu medication as a prospective anti-Alzheimer's medication and indicates the need for in-depth *in vivo* investigation in the future.

Keywords Kangen-karyu, Alzheimer's disease, cholinesterase, BACE1, GSK3β

1. Introduction

With an increase in the elderly population in developing countries, the prevalence of Alzheimer's disease (AD) is rapidly increasing (*1*). Thirty-six million people suffer from AD worldwide and almost 50% of the cases of dementia are due to AD (*2*). Therefore, AD has become a major health problem and an economic burden for health systems. It is characterized by the occurrence of extracellular amyloid plaque deposits and neurofibrillary tangles of the microtubule-binding protein tau in the central nervous system (*3,4*), and clinical symptoms include compromised memory as well as cognition, orientation, and several emotional disturbances (*5*).

AD is a complex neuronal disease involving various factors. Although the exact etiology is still unclear, studies on the AD brain and AD animal models have proposed several hypotheses regarding AD: (a) Cholinergic hypothesis – loss of cholinergic neurotransmission in the cerebral cortex due to an increase in cholinesterase activity and degeneration of cholinergic neurons in the basal forebrain deteriorate cognition (*6,7*). (b) Amyloid hypothesis – proteolytic cleavage of the amyloid precursor protein (APP) by secretases forms amyloid-beta (Aβ) fibrils, and the accumulated Aβ amyloid fibrils develop into senile plaque, causing neurotoxicity, tauopathy, neuronal cell death, and neurodegeneration (*8,9*). (c) Tau hypothesis – tau is a microtubule-associated protein in axons that regulates the stability of tubulin assemblies; however, aggregation in a hyperphosphorylated state impairs axons of neurons (tauopathy) (*10,11*). (d) Glycogen synthase kinase 3β (GSK3β) hypothesis – hyperactive GSK3 leads to tau hyperphosphorylation, increased Aβ production, and memory impairment (*12,13*). Therefore, since there are several causes of AD, traditional Chinese medicine (TCM) or its combination therapy with various medicinal properties rather than a single agent mostly used in current AD therapy has the potential to be an effective treatment for AD.

TCM has been widely used in China for thousands of years. Recently, TCM has established its position in the Western world. Some TCM have been reported to be either ineffective or lethal due to hepatotoxicity at higher concentrations or herb-drug interactions (*14*). However, the majority of TCM, through controlled clinical trials, have been proven to be safe and effective (*15*). Herein, we systematically reviewed anti-AD activity of a herbal mixture Kangen-karyu (Guan-

Yuan-Ke-Li in Chinese has been developed in Japan *via* partial modification of the herbal constituents of the Chinese herbal prescription Guan-Xin No. 2), and its individual components (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma), particularly focusing on cholinergic, amyloid, tau, and GSK3β hypotheses of AD treatment. We also discuss *in vivo* studies on Kangen-karyu and its safety, highlighting its potential in neuronal drug discovery.

2. Cholinesterases as molecular targets of Kangen-karyu

Acetylcholine (ACh) is a neurotransmitter of all cholinergic neurons in the central and peripheral nervous systems that modulates neural functions in attention, learning, memory, stress responses, wakefulness and sleep, and responses to sensory information (*16*). Cholinergic neurotransmission relies on ACh synthesis, storage, transportation, and degradation. Acetylcholinesterase (AChE) is an enzyme responsible for ACh degradation *via* hydrolysis to acetate and choline. It is one of the most kinetically efficient enzymes because each molecule can hydrolyze 5,000 molecules of ACh/second (*17*). So, it is a highly effective therapeutic target for symptomatic relief in AD patients because cholinergic deficit is a consistent finding in early AD.

Impairment of brain AChE levels in diabetes is one of the reasons for diabetes-associated cognitive decline (*18*). In a previous study, dried powder of a boiled water extract of Kangen-karyu attenuated cognitive deficit in diabetic mice (*19*). Furthermore, the study was conducted *in vivo* using a cognitive deficit-diabetic mouse model by performing behavioral experiments, and evaluating the cholinergic marker protein choline acetyltransferase (ChAT) and muscarinic acetylcholine (M_1, M_3, and M_5) receptors in the hippocampus (*19*). In a behavioral study, 18-week-old *db/db* mice with a diabetic insult showed a marked decrease in spatial learning performance in terms of the escape latency compared with the same aged-matched non-diabetic mice. However, daily treatment of *db/db* mice with 200 mg/kg Kangen-karyu significantly and dose-dependently improved the spatial learning performance of old *db/db* animals in the training test. The administration of 100 mg/kg/day of Kangen-karyu also led to significant improvement in learning performance compared with a vehicle-treated group. In Western blot analysis, a vehicle-treated old *db/db* group showed significantly lower levels of ChAT, M_1 receptor, M_3 receptor, and M_5 receptor. However, the daily oral administration of Kangen-karyu extract (100 and 200 μg/mL) led to a significant increase in ChAT, M_1 receptor, M_3 receptor, and M_5 receptor levels. Decreases in levels of ACh, ChAT, and muscarinic and nicotinic ACh receptors are associated with cholinergic hypofunction with cognitive deficits (*20-22*), and the ability of Kangen-karyu to increase the reduced level of these markers in cognitive deficit mouse models demonstrates a neuroprotective role.

To our knowledge, there have been no reports on cholinesterase inhibition by the component Cnidii Rhizoma. From this component, ferulic acid, sinapic acid, 5-hydroxy ferulic acid, and chlorogenic acid have been reported (*23*). A previous study reported the effect of sinapic acid on the basal forebrain of an ibotenic acid-treated rat model (*24*). In that study, treatment of rats with ibotenic acid decreased ACh levels in the parietal and frontal cortices and ChAT activity in the parietal cortex. However, pretreatment of rats with sinapic acid (3 and 10 mg/kg) reversed the effect of ibotenic acid in a dose-dependent manner. A dose of 10 mg/kg of sinapic acid led to significant retention of the ACh levels and ChAT activity in the basal forebrain.

In a study conducted to identify novel AChE inhibitors derived from Salviae Miltiorrhizae Radix (*25*), two abietane diterpene dihydrotanshinone and cryptotanshinone showed promising inhibition of bovine erythrocyte AChE with IC_{50} values of 1.0 and 7.0 μM, respectively. However, tanshinone I and tanshinone IIA displayed weak inhibitory effects. Similarly, for human cloned cholinesterase (*26*), dihydrotanshinone and cryptotanshinone showed mixed non-competitive inhibition of AChE with IC_{50} values of 0.89 and 4.67 μM, respectively. For human butyrylcholinesterase (BChE), the inhibition mode was uncompetitive with IC_{50} values of 6.66 μM for cryptotanshinone and 5.51 μM for dihydrotanshinone. Also, Wong and colleagues (*26*) performed molecular docking studies to explore the binding mechanism of these diterpenes with human cloned cholinesterases. In the presence of human AChE, these compounds bound to the active-site gorge lining Trp86, Tyr124, and Tyr337 with different orientations. The penta ring of dihydrotanshinone faced the bottom of the gorge while cryptotanshinone faced the opposite direction – towards the gorge mouth. At the catalytic site, both compounds interacted with the prime catalytic triad residues Ser203 and His447. This suggested that additional interaction of dihydrotanshinone with Tyr337 and Gly120 *via* H-bonds plays a vital role due to its potency compared with cryptotanshinone. Interestingly, the docking of these diterpenes with hBChE led to a similar binding configuration involving aromatic residues, Trp430, Phe329, and Tyr332.

The crude extract of Paeoniae Radix and its major component, paeoniflorin, were previously reported to have beneficial effects on spatial cognitive deficits caused by the dysfunction of central cholinergic systems and aging in rodents (*27,28*). Also, in a previous report, an ethanol extract of Paeoniae Radix demonstrated good inhibition of AChE and BChE with IC_{50} values of 25.04 and 10.59 μg/mL, respectively (*29*).

3. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) as a molecular target of Kangen-karyu

BACE1 is a type 1 transmembrane aspartyl protease that is involved in the generation of Aβ peptides by neurons through proteolytic cleavage of APP (*30*). Cleaving of APP by β-secretase at the NH_2-terminus of Aβ to release a soluble ~100-kD NH_2-terminal fragment and a membrane-bound 12-kD COOH-terminal fragment initiates Aβ formation. The progressive formation of insoluble amyloid plaque and vascular deposits of Aβ peptide is a characteristic event in AD (*31*).

The effect of Kangen-karyu on BACE1 activity has yet to be reported. We performed a comparative study on BACE1 inhibition with a boiled water extract of Kangen-karyu and its components (Table 1) (data not published). The boiled water extract of Kangen-karyu demonstrated moderate inhibition. Among the fractions, moderate inhibition was also observed with a boiled water extract of Salviae Miltiorrhizae Radix and Cyperi Rhizoma followed by mild inhibition by Paeoniae Radix, Cnidii Rhizoma, and Carthami Flos. No noticeable inhibition was observed with a boiled water extract of Aucklandiae Radix.

Furthermore, depending upon the activity of individual components of Kangen-karyu, we further studied Salviae Miltiorrhizae Radix. We performed a comparative study on BACE1 inhibition by MeOH and water extracts of Salviae Miltiorrhizae Radix along with solvent-soluble fractions (*n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, and water fractions) from each extract (data not published). As shown in Table 2, the MeOH extract of Salviae Miltiorrhizae Radix showed moderate inhibition of BACE1 with an IC_{50} value of 114.82 ± 3.00 μg/mL. Among the fractions of MeOH extract, *n*-hexane and CH_2Cl_2 fractions showed promising inhibition followed by EtOAc and *n*-BuOH fractions. However, the H_2O fraction had a mild inhibitory effect. Interestingly, the water extract of Salviae Miltiorrhizae Radix showed good inhibition (Table 3). Among the fractions, the EtOAc fraction demonstrated the most promising inhibition (IC_{50} = 0.20 ± 0.07 μg/mL), followed by CH_2Cl_2 (IC_{50} = 6.11 ± 0.53 μg/mL), *n*-hexane (IC_{50} = 11.83 ± 0.09 μg/mL), *n*-BuOH (IC_{50} = 49.32 ± 2.62 μg/mL), and H_2O (IC_{50} = 64.77 ± 7.59 μg/mL) fractions, respectively.

From a structural viewpoint, Salviae Miltiorrhizae Radix comprises water-soluble polyphenolic compounds – danshensu (3, 4-dihydroxyphenyllactic acid), salvianolic acid A, B, and C, protocatechuic aldehyde, and lipophilic compounds – tanshinones. HPLC analysis of a water extract of Salviae Miltiorrhizae Radix revealed tanshinone II and salvianolic acid B as dominant components (*32*). Because of the complex AD pathology, the neuroprotective components tanshinones and polyphenolics – salvianolic acids might be effective in treating AD (*33*). In our previous study (*34*), we evaluated the BACE1 inhibitory effect of tanshinones and salvianolic acid derivatives from Salviae Miltiorrhizae Radix along with enzyme kinetics and molecular docking simulations. Among the tested compounds, deoxyneocryptotanshinone and salvianolic acid C exhibited mixed modes of BACE1 inhibition with IC_{50} values of 11.53 ± 1.13 and 9.18 ± 0.03 μM, respectively. Similarly, salvianolic acid A inhibited the enzyme activity competitively with an IC_{50} value of 13.01 ± 0.32 μM. Most of the other tanshinones had moderate inhibitory effects with IC_{50} values ranging from 30 to 50 μM. Salvianolic acid A and C demonstrated higher binding affinity at the active catalytic site of BACE1 involving H-bond interaction with conserved aspartic acid residues Asp228 and

Table 1. BACE1 inhibitory potentials of water extract of Kangen-karyu and its constituents

Sample	IC_{50} values[a]
Kangen-karyu	77.40 ± 4.58
Aucklandiae Radix	> 400
Carthami Flos	233.34 ± 0.05
Cnidii Rhizoma	147.09 ± 0.93
Cyperi Rhizoma	91.16 ± 2.21
Paeoniae Radix	143.57 ± 1.79
Salviae Miltiorrhizae Radix	89.84 ± 1.87
Quercetin[b]	10.49 ± 0.28[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Table 2. BACE1 inhibitory potentials of MeOH extract from Salviae Miltiorrhizae Radix and its various fractions

Sample	IC_{50} values[a]
MeOH extract	114.82 ± 3.00
n-Hexane fraction	11.79 ± 0.03
CH_2Cl_2 fraction	12.06 ± 0.58
EtOAc fraction	74.05 ± 0.64
n-BuOH fraction	115.79 ± 9.34
H_2O fraction	>150
Quercetin[b]	9.26 ± 0.36[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Table 3. BACE1 inhibitory potentials of H_2O extract from Salviae Miltiorrhizae Radix and its various fractions

Sample	IC_{50} values[a]
H_2O extract	17.82 ± 0.35
n-Hexane fraction	11.83 ± 0.09
CH_2Cl_2 fraction	6.11 ± 0.53
EtOAc fraction	0.20 ± 0.07
n-BuOH fraction	49.32 ± 2.62
H_2O fraction	64.77 ± 7.59
Quercetin[b]	9.26 ± 0.36[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Asp32. Prime interacting residue (Ser10) was observed for deoxyneocryptotanshinone and salvianolic acid C at the allosteric site. Oxygen groups of Ser229 and Glu310 interacted with the oxygen O11, O12, O15, and O16 of salvianolic acid B *via* hydrogen bonding interaction. The oxygen groups O6 and O15 of salvianolic acid C formed two H-bonds with Gly13 while O3, O6, and O7 interacted with Lys9, Gln304, and Asp318 *via* hydrogen-bonding. These findings reveal that the hydroxyl moieties in salvianolic acids play crucial roles in BACE1 interaction. Structure-activity relationships within caffeic acid derivatives reveal that the phenolic –OH group has a crucial effect on BACE1 inhibition. Activity was enhanced with an increase in phenolic –OH groups in rosmarinic acid (IC_{50}: 29.77 ± 0.70 μM) and magnesium lithospermate (IC_{50}; 30.35 ± 2.67 μM) compared with caffeic acid (IC_{50}; > 200 μM). The number of phenolic –OH groups in magnesium lithospermate is higher than in rosmarinic acid; however, the activity was similar. The arrangement of alkoxy groups and presence of magnesium might be responsible for this effect.

4. GSK3β as a molecular target of Kangen-karyu

GSK3 is one of the prime targets of AD and is responsible for the generation of paired helical filaments-tau, a major component of neurofibrillary tangles in the brain (*35*). It is a multifunctional proline-directed serine/threonine protein kinase for glycogen synthase phosphorylation involved in diverse biological processes (*36*). Cumulative evidence identifies glycogen synthase kinase as a potential target for neuroprotection because it contributes to the AD-associated hyperphosphorylation of tau and tau protein as a widely recognized substrate of GSK3 (*37*). The reduction of aberrant over-activity of this enzyme decreases various aspects of AD pathology (*38*). Two isomeric forms of GSK, GSK3α and GSK3β, share 98% homology in the catalytic domain, have similar biochemical properties, and are ubiquitously expressed in cells and tissues. Phosphorylation of serine residue (Ser21 in GSK3α and Ser9 in GSK3β) inhibits GSK3 (*36*).

In our previous study conducted to evaluate anti-AD activity *in vitro via* the GSK3 enzyme, boiled water extract of Kangen-karyu demonstrated potent inhibition of GSK3β with an IC_{50} value of 17.05 ± 1.14 μg/mL, as shown in Table 4 (*39*). Also, water extracts of all individual components showed good inhibition with IC_{50} values ranging from 7.77 to 93.61 μg/mL. Water extract of Salviae Miltiorrhizae Radix showed promising inhibition among the components followed by Cyperi Rhizoma. Other components showed mild inhibition with the following potency order: Paeoniae Radix > Cnidii Rhizome > Aucklandiae Radix > Carthami Flos (Table 4). Polar constituents (rosmarinic acid, magnesium lithospermate B, salvianolic acid A, salvianolic acid B, and salvianolic acid C) that were reported from water extract of Salviae Miltiorrhizae Radix were tested for GSK3β inhibition. The results demonstrated that these polar constituents inhibited GSK3β with IC_{50} values ranging from 6.97 to 135.5 μM. Among them, salvianolic acid B was the most potent ATP-competitive inhibitor of GSK3β with the lowest IC_{50} value (6.97 ± 0.96 μM). With IC_{50} values of approximately 30 μM, salvianolic acid A, salvianolic acid B, and magnesium lithospermate B showed good inhibition followed by the moderate activity of rosmarinic acid (IC_{50}: 135.35 ± 4.69 μM) and mild inhibition by caffeic acid (IC_{50}: 425.01 ± 7.61 μM). Although direct evidence of increased GSK3 activity in AD at present is still limited, studies have clearly demonstrated upregulated GSK3 expression in the hippocampus (*40*) and peripheral lymphocytes (*41*) of AD patients.

Table 4. GSK3β inhibitory potentials of water extract of Kangen-karyu and its constituents

Sample	IC_{50} values[a]
Kangen-karyu	17.05 ± 1.14
Aucklandiae Radix	85.04 ± 6.32
Carthami Flos	93.61 ± 3.99
Cnidii Rhizoma	66.74 ± 2.05
Cyperi Rhizoma	20.68 ± 2.50
Paeoniae Radix	62.51 ± 1.89
Salviae Miltiorrhizae Radix	7.77 ± 1.38
Luteolin[b]	2.18 ± 0.13[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SEM. [b]Used as positive control. [*]Values are expressed in μM.

5. Future perspective and conclusion

Although there are reports on anti-AD activity of some compounds that are contained in either of the six components of Kangen-karyu, a detailed and systematic pharmacological study of Kangen-karyu and its individual components is lacking. Generally, traditional medicines are formulae (a mixture of herbs/herbal components), serving as combination therapy. As such, it is difficult to determine the precise pharmacology of the formulae because they can have multiple targets and might also have multiple actions along with synergistic, additive, and/or antagonistic effects. Owing to this and to search for therapeutics pursuing the 'one drug-fits-all' concept, the utility of combination therapy has been highlighted (*42*). This necessitates in-depth pharmacology of Kangen-karyu for the management of AD.

The present review highlights the possible role of Kangen-karyu in the management of AD *via* cholinesterase, BACE1, and GSK3β inhibition.

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