Towards a bioinformatics analysis of anti-Alzheimer’s herbal medicines from a target network perspective

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Abstract
With the growth of aging population all over the world, a rising incidence of Alzheimer’s disease (AD) has been recently observed. In contrast to FDA-approved western drugs, herbal medicines, featured as abundant ingredients and multi-targeting, have been acknowledged with notable anti-AD effects although the mechanism of action (MOA) is unknown. Investigating the possible MOA for these herbs can not only refresh but also extend the current knowledge of AD pathogenesis. In this study, clinically tested anti-AD herbs, their ingredients as well as their corresponding target proteins were systematically reviewed together with applicable bioinformatics resources and methodologies. Based on above information and resources, we present a systematically target network analysis framework to explore the mechanism of anti-AD herb ingredients. Our results indicated that, in addition to the binding of those symptom-relieving targets as the FDA-approved drugs usually do, ingredients of anti-AD herbs also interact closely with a variety of successful therapeutic targets related to other diseases, such as inflammation, cancer and diabetes, suggesting the possible cross-talks between these complicated diseases. Furthermore, pathways of Ca\(^{2+}\) equilibrium maintaining upstream of cell proliferation and inflammation were densely targeted by the anti-AD herbal ingredients with rigorous statistic evaluation. In addition to the holistic understanding of the pathogenesis of AD, the integrated network analysis on the MOA of herbal ingredients may also suggest new clues for the future disease modifying strategies.

Keywords: Alzheimer’s disease (AD); network pharmacology; herbal medicine; therapeutic target

INTRODUCTION
Most complex diseases, such as cancer, inflammation and neurodegenerative disorders, are usually caused by multiple genes or their products. The efficacy of single targeting therapies has often been found limited due to insufficient understanding of the complex...
network essence of these diseases [1]. Alzheimer’s
disease (AD) is one of such which is clinically char-
cacterized by an age-dependent memory dysfunction
together with cognitive deterioration [2]. Although
the etiology of AD is still waiting to be revealed,
multiple pathogenetic factors have been identified
to be involved in this disease. Animal model studies
have shown that brain accumulation of amyloid beta
(Aβ) and neurofibrillary tangles (NFTs) are essential
for the development of AD [3]. In addition, prom-
inent activation of inflammatory processes, the innate
immune response, mitochondrial dysfunction, oxi-
dative stress and neuron apoptosis have been
observed in AD progression as well [4].

Currently, there are mainly two types of
FDA-approved drugs. One is cholinesterase inhibi-
tors (ChEIs) designed based on therapeutic target of
cholinesterase. There are four approved cholinester-
ase inhibitors [5]: Tacrine, Donepezil, Rivastigmine
and Galantamine. They are used for the treatment of
mild to moderate AD, and postulated to exert therapeu-
tic effect by enhancing cholinergic function [6].
The other one is N-methyl-D-aspartate antagonists
[7] specifically targeted to NMDA receptors. The
only approved NMDA receptor antagonist is
Memantine [5], which is applied in the treatment
of moderate to severe AD. As it is reported that
these approved drugs only help to relieve the symp-
toms of AD patients rather than producing efficient
disease-modifying effects due to their monotonous
therapeutic function [4], new AD therapies are in
urgent need. In the past few years, many small mol-
ecules or bioproducts are under development for the
treatment of AD, including chemical drugs and anti-
body therapeutics.

Among these drugs, herbal medicines, derived
from thousands of years of application, are especially
promising in the development of AD therapy. As
being supported by cellular and animal model stud-
ies, herbal medicines were reported to be effective
on AD from multiple pathways. Several herbal ex-
tracts, such as the Ginkgo biloba extract EGb761 and
Salvia officinalis extracts have been found with a com-
bination of anti-oxidative, anti-apoptosis, neuropro-
ective and neuromodulatory effects [8–11]. The use
of herbal medicines in the treatment of AD is attract-
ing more and more attention [12, 13], and some of
which have even advanced to clinical trials. How-
ever, their anti-AD mechanism is not yet well
understood. Several questions are still remained to be
solved: what are the ingredients of these herbal
medicines and their target proteins? Are they over-
lapping with therapeutic targets of FDA-approved
western drugs? What pathways are herbal medicines
involving in disease treatment? What is the mechan-
istic difference between herbal medicines and west-
ern drugs? Studying these questions may not only
reveal the MOA of anti-AD herbs but also extend
the domain knowledge of AD pathogenesis.

In this paper, we extensively reviewed current
available in silico methods for anti-AD drug research
and development. Based on the survey of these bio-
informatics methodologies, we described a frame-
work to construct an easily-interpretable map that
could facilitate elucidating (MoA) for anti-AD
herbal medicines. First, the clinically supported
anti-AD herbs, their ingredients, as well as the
target proteins were collected. Then, they were stu-
died as compared to FDA-approved anti-AD drugs
in the context of target network. Finally, a holistic
understanding of the molecular mechanism respon-
sible for the anti-AD effects of the herbal medicines
was presented, and the molecular mechanism
involved in the AD was also refreshed and extended
from the observed action of those herb medicines.

**COMPUTATIONAL STRATEGIES
FOR ANTI-AD DRUG RESEARCH**

During the past decades, computational methods
have facilitated the exploration of anti-AD drugs
with the abundant bioactivity information. They
could mainly be classified into two categories, i.e.:
‘single-target’-directed and ‘multi-target’-directed
strategy.

**‘Single-target’ strategy of anti-AD drugs**

Several in silico methods have been frequently em-
ployed into ‘single-target’ anti-AD drug research
based on the conception of ‘therapeutic target’. For
example, docking approaches, molecular dynamics
studies, quantum mechanical studies, and other
methods that could exhibit quantitative structure–
activity relationships (QSAR) have been applied
to facilitate the understanding of cholinesterase,
and to design new cholinesterase inhibitors
[14–18]. Furthermore, ligand-based computational
approaches have also been used to identify inhibitors
for other attractive targets for AD, such as β-Secretase [19–21] and γ-Secretase [22].
In recent years, the system-oriented design of ‘multi-target’ anti-AD drugs is an attractive approach with increasing interests, as AD is a complex disease involving multiple pathogenetic factors. Besides the computational methods used in ‘single-target’ anti-AD drug design, several new approaches could facilitate the development of ‘multi-target’ anti-AD drugs with integration of various sources of information, such as microarrays, literature mining-data, protein–protein interactions (PPIs), and so on. These methods were summarized in Table 1.

There are various AD-related microarray gene-expression profiles, and many of them have been well-collected in the Connectivity Map (cMAP) [23], as well as the newly published searchable platform independent expression database (SPIED) [24]. Different methods have been applied to explore the microarray gene-expression dataset of AD, including unsupervised machine learning approaches [25], integer linear programming model [26] and network analysis [27, 28]. Potential pathways and therapeutic targets for AD were also identified in these studies.

Nevertheless, the gene-expression experiments are expensive and time-consuming, and often reported in disagreement with low-throughput experiments. Hence, alternative methods without requiring high-throughput microarray data are attractive as well. For example, two newly published methods were proposed based on the interacting profiles of AD drugs and could help to uncover their therapeutic mechanisms by incorporating the ‘off-targets’. Among them, one is the AD-specific drug–protein connectivity map [29] built with protein interaction networks and literature mining. The other one is the AD drug-oriented chemical–protein interactome (CPI) [30], which was constructed on the basis of the relations between 10 drug molecules and 401 human protein pockets derived from the application of DOCK program. Furthermore, a network was integrated with published PPI data to study the functional relevance among AD-related proteins and the relationships among them [31, 32]. This network analysis provides a systematic view of AD pathology, as it could help to find not only important AD-related proteins, but also important pathways involving in AD.

### FRAMEWORK FOR THE ANTI-AD MECHANISM ANALYSIS OF HERBAL MEDICINES

According to the review of current in silico methods for anti-AD drug discovery, there seems no published work for the holistic understanding of the molecular mechanism for anti-AD drugs. In this section, we considered to provide a low-cost and effective way to explore the anti-AD mechanism at a system level. Specifically, we focused on anti-AD herbs and the network pharmacology analysis technologies. It should be noted that network analysis is an increasingly important approach in uncovering the mode of drug action, which has been well-illustrated in several articles [33–35]. Especially, this system-based network technology is useful in revealing the
complex modulation essence of herbal medicines as concluded in the review of Jing Zhao et al. [36]. As a result, we attempt to (i) systematically collect and review anti-AD herbs, their ingredients, and the target proteins from public databases and literatures, and (ii) study the anti-AD mechanism with the application of network analysis. This two-part framework was shown in Figure 1.

Mining of anti-AD herbs, their ingredients and the corresponding target proteins

The main purpose of this part is to answer the following questions: what are the ingredients of herbal medicines and their target proteins? Are they overlapping with therapeutic targets of FDA-approved western drugs?

**Anti-AD herbs**

We have performed a large-scale text mining of PubMed and the clinical trial database (www.Clinicaltrials.gov, currently contains 119 213 trials from 178 countries), and for the first time manually extracted the available promising anti-AD herbs from the English literatures (from 1995 to 2011) with the keywords ‘herbal medicine’ and ‘Alzheimer’s’. Among them, Ginkgo biloba, Huperzia serrata, Melissa officinalis and Salvia officinalis are found to be the top well studied ones in the large volume of AD-related articles as shown in Table 2. It can be seen from literatures that different herbs have been studied to different extent. Articles relating Ginkgo biloba to AD are the most abundant (233), probably because Ginkgo biloba has been studied for a long time and extensive papers have been accumulated (2357 articles). This herb could significantly improve attention and memory performance of patients in a phase III clinical trial [37]. The efficacy of Ginkgo biloba was even comparable to the results of patients treated with donepezil, a FDA-approved drug for mild to moderate AD [37].

As these herbs have been studied to different extent, a parameter is introduced to balance this bias and further assess the association between them and AD. The parameter is calculated as the (AD-herb-related papers)/(herb-related papers) ratio. P-value was applied to measure how improbable by chance it is to observe a certain level of co-occurrences of each herb and AD in at least $k$ articles [38]:

$$P = 1 - \sum_{i=0}^{k-1} f(i) = 1 - \sum_{i=0}^{k-1} \binom{K}{i} \frac{(N-K)}{n-i} \binom{N}{n},$$

where $N$ is the total number of papers in PubMed (19 432 858 articles, as given by GoPubMed), $K$ is the number of papers associate with AD (52 535 articles, as given by GoPubMed), $n$ is the volume about one single herb, $k$ is the number of papers about the effects of corresponding herb on AD. GoPubMed was used to get the value of $N$, $K$, $n$ and $k$. P-value indicates the significance of relevance between each herb and AD [39] (significant when $P$-value < 0.01).

As a result, Huperzia serrata obtained the highest ratio (35.71%; with $P < 0.01$), indicating that anti-AD may be one of the major therapeutic effects found so far for this herb. Ginkgo biloba (9.89%; with $P < 0.01$) is second to Huperzia serrata, and then followed by Salvia officinalis (3.99%; with $P < 0.01$) and Melissa officinalis (2.29%; with $P < 0.01$).

Furthermore, different stages of clinical trials for these four herbs were summarized from either clinical trial databases or PubMed, as shown in Table 3. This suggested that these herbal medicines are promising in the field of anti-AD drug discovery, as random and double-blinded clinical studies gave positive results. Among the four herbs, Ginkgo biloba...
was the most frequently studied one as 12 related clinical trials can be found. Only 4 of the 12 trials showed no significant differences from the placebo groups, and all the other 8 studies have provided supportive evidence for anti-AD effects of *Ginkgo biloba*. For *Huperzia serrata*, most of its clinical trials were focused on the component named Huperzine A and significant improvement has been observed in four trials. As to *Melissa officinalis* and *Salvia officinalis*, there was only one trial for each.

**Anti-AD herb ingredients**

In this study, the compounds contained in each herb were collected from Traditional Chinese Medicine Information Database (TCM-ID) [45], ‘Pharmacopoeia of People’s Republic of China’ and PubMed. It is noted that not all compounds were extensively studied, and only those with literature support of target proteins are remained. Finally, 10 compounds were collected from the four herbs. The significance of their correlations with AD were also measured with the *P*-value introduced in the ‘Anti-AD herbs’ part, and were summarized in Table 2. It is not surprising to observe that *P*-values for the majority of the ingredients are not significant, as herbal medicines are usually used in the form of multiple components. Among these ingredients, Huperzine A from *Huperzia serrata* has the highest correlation with AD (25.81% and 80 articles), and this correlation is significant as measured by *P*-value. Actually, results of the most recent Phase II clinical trial have indicated the significant cognitive enhancement of Huperzine A for mild to moderate AD patients [12]. The activity of this ingredient was reported to be stronger than another ChEI galantamine [46]. Phase III and Phase IV trials of Huperzine A are also anticipated, as indicated by the clinical trial database.

**Target proteins of anti-AD herb ingredients**

Information of target proteins for herbal ingredients was identified from HIT [47], a well-known herb ingredient target database (http://lifecenter.sgst.cn/hit/). A total of 221 direct targets of 586 herbal compounds have been collected in this database. Totally 34 target proteins were retrieved for the 10 anti-AD herbal ingredients (Table 4). The interactions between the compounds and targets have been classified into three categories, i.e. activation, inhibition and neutral binding of the target proteins.

Then, to assess the relationship between these target proteins and AD:

First, the 34 target proteins were regarded as a whole to evaluate the distance between them and known ‘AD-related proteins’ in the background of a genome-wide human PPI network. If the distances between the herbal targets and ‘AD-related proteins’ are significantly shorter than those of the random counterparts in the context of human PPI, a close
Table 3: Information for clinical trials of anti-AD herbs

<table>
<thead>
<tr>
<th>Herbal name</th>
<th>Study</th>
<th>Sample</th>
<th>Experimental design</th>
<th>Intervention</th>
<th>Outcome measure</th>
<th>Conclusion</th>
<th>Information source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgo biloba</td>
<td>Phase III clinical trial</td>
<td>Age: ≥75</td>
<td>prevention; randomized; double blind; placebo control; parallel assignment; safety/ efficacy study</td>
<td>120 mg/2 days G. biloba; placebo</td>
<td>ADAS-cog; CDR</td>
<td>Negative</td>
<td>Clinical trial database (NCT00010803)</td>
</tr>
<tr>
<td></td>
<td>Status: normal cognition (n=2587) or MCI (n=482)</td>
<td></td>
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</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Area: Germany</td>
<td>Time: 24 weeks</td>
<td>Number: 216</td>
<td>double blind; placebo control; randomized; multicenter</td>
<td>240 mg/day G. biloba; placebo</td>
<td>ADAS-cog; SKT</td>
<td>Positive</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Area: US</td>
<td>Time: 26 weeks</td>
<td>Number: 309</td>
<td>randomized; double blind; placebo control; parallel group; multicenter</td>
<td>120 mg/day G. biloba; placebo</td>
<td>ADAS-Cog</td>
<td>Positive</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Area: Germany</td>
<td>Time: 3 months</td>
<td>Age: 50−80</td>
<td>randomized; double blind; placebo control</td>
<td>240 mg/day G. biloba; placebo</td>
<td>SKT; ADAS</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Status: mild to moderate AD</td>
<td>Number: 20</td>
<td></td>
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<tr>
<td>Ginkgo biloba</td>
<td>Area: The Netherlands</td>
<td>Time: 24 weeks</td>
<td>Number: 214</td>
<td>double blind; randomized; placebo control; parallel group; multicenter</td>
<td>240 mg/day G. biloba; 160 mg/day G. biloba; placebo</td>
<td>NAI-WL; NAI-ZN-G</td>
<td>Negative</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Area: US</td>
<td>Time: 26 weeks</td>
<td>Number: 513</td>
<td>randomized; placebo control; double blind; parallel group; multicenter</td>
<td>240 mg/day G. biloba; 120 mg/day G. biloba; placebo</td>
<td>ADAS-cog; ADCS-CGIC</td>
<td>Negative</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Area: UK</td>
<td>Time: 6 months</td>
<td>Number: 176</td>
<td>randomized; pragmatic; double blind; community based; parallel group</td>
<td>120 mg/day G. biloba; placebo</td>
<td>ADAS-Cog</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Status: early stage dementia</td>
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(continued)
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<tr>
<th>Herbal name</th>
<th>Study</th>
<th>Sample</th>
<th>Experimental design</th>
<th>Intervention</th>
<th>Outcome measure</th>
<th>Conclusion (positive or negative evidence for the herbal medicine in AD treatment)</th>
<th>Information source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgo biloba</td>
<td>Area: Italy</td>
<td>Age: 50–80 Status: mild to moderate dementia</td>
<td>randomized; placebo control; double blind placebo control</td>
<td>160 mg/day G. biloba; 5 mg/day donepezil; placebo</td>
<td>MMSE</td>
<td>Positive</td>
<td>PubMed:16930364</td>
</tr>
<tr>
<td></td>
<td>Time: 24 weeks</td>
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<tr>
<td></td>
<td>Area: US</td>
<td>Number: 168 Status: primarily visual-constructual impairment or predominant verbal deficits or impaired in both cognitive domains.</td>
<td>randomized; double blind placebo control</td>
<td>120 mg/day G. biloba; placebo</td>
<td>ADAS-Cog</td>
<td>Positive</td>
<td>PubMed:13130389</td>
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<tr>
<td></td>
<td>Time: 52 weeks</td>
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<tr>
<td>Ginkgo biloba</td>
<td>Area: US</td>
<td>Number: 309 Status: AD or multi-infarct dementia</td>
<td>multicenter; randomized; double blind placebo control</td>
<td>120 mg/day G. biloba; placebo</td>
<td>ADAS-cog; ADCS-CGIC</td>
<td>Positive</td>
<td>PubMed:9343463</td>
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<tr>
<td></td>
<td>Time: 52 weeks</td>
<td></td>
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<tr>
<td>Hup A</td>
<td>Phase II clinical trial</td>
<td>Number: 150</td>
<td>randomized; multicenter; double blind placebo control</td>
<td>200 μg/2 days Hup A; 400 μg/2 days Hup A; placebo</td>
<td>ADAS-cog</td>
<td>Positive</td>
<td>Clinical trial database: NCT00083590</td>
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<tr>
<td></td>
<td>Area: US</td>
<td>Time: 24 weeks</td>
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<tr>
<td>Hup A</td>
<td>Area: China</td>
<td>Number: 50 Status: AD</td>
<td>randomized; double blind placebo control; parallel placebo control; placebo control</td>
<td>200 μg/day Hup A; placebo</td>
<td>MMSE</td>
<td>Positive</td>
<td>PubMed:8701750</td>
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<tr>
<td></td>
<td>Time: 8 weeks</td>
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<tr>
<td>Hup A</td>
<td>Area: China</td>
<td>Number: 60 Status: AD</td>
<td>randomized; double blind placebo control; parallel placebo control; parallel placebo control; placebo control</td>
<td>200 mg/day Hup A; placebo</td>
<td>psychological evaluations</td>
<td>Positive</td>
<td>PubMed:10678137</td>
</tr>
<tr>
<td></td>
<td>Time: 60 days</td>
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<tr>
<td>Hup A</td>
<td>Area: China</td>
<td>Number: 202 Status: AD</td>
<td>randomized; double blind placebo control; parallel placebo control; parallel placebo control; placebo control</td>
<td>400 mg/day Hup A; placebo</td>
<td>ADAS-Cog; MMSE</td>
<td>Positive</td>
<td>PubMed:12181083</td>
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<tr>
<td></td>
<td>Time: 12 weeks</td>
<td></td>
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<tr>
<td>Melissa officinalis</td>
<td>Area: Tehran, Iran</td>
<td>Age: 65–80 Status: mild to moderate AD</td>
<td>randomized; double blind placebo control; parallel assignment placebo control</td>
<td>Melissa officinalis; placebo</td>
<td>ADAS-cog; CDR</td>
<td>Positive</td>
<td>PubMed:12810768</td>
</tr>
<tr>
<td></td>
<td>Time: 4 months</td>
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<tr>
<td>Salvia officinalis</td>
<td>Area: Tehran, Iran</td>
<td>Age: 65–80 Status: mild to moderate AD</td>
<td>randomized; double blind placebo control; parallel assignment placebo control</td>
<td>Salvia officinalis; placebo</td>
<td>ADAS-cog; CDR</td>
<td>Positive</td>
<td>PubMed:12605649</td>
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<tr>
<td></td>
<td>Time: 4 months</td>
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<table>
<thead>
<tr>
<th>Target name</th>
<th>Target name (abbreviation)</th>
<th>Uniprot.ID</th>
<th>Gene.ID</th>
<th>Compound</th>
<th>Interacting type</th>
<th>State of therapeutic target</th>
<th>Herb</th>
<th>PubMed.ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetylcholinesterase</td>
<td>AChE</td>
<td>P22303</td>
<td>43</td>
<td>Huperzine A; Huperzine B</td>
<td>i; i</td>
<td>Successful target (AD)</td>
<td><em>Huperzia serrata</em></td>
<td>16364207; 16364207</td>
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<tr>
<td>2. Glutamate[NMDA] receptor</td>
<td>NMDAR</td>
<td>Q05586</td>
<td>2902</td>
<td>Huperzine A</td>
<td>i</td>
<td>Successful target (AD)</td>
<td><em>Huperzia serrata</em></td>
<td>11920920</td>
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<tr>
<td>3. Cholinesterase</td>
<td>BCHE</td>
<td>P06276</td>
<td>590</td>
<td>Huperzine A; Huperzine B</td>
<td>i; i</td>
<td>Successful target (AD)</td>
<td><em>Huperzia serrata</em></td>
<td>16364207; 16364207</td>
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<td>4. Epidermal growth factor receptor</td>
<td>EGFR</td>
<td>P00533</td>
<td>1956</td>
<td>Quercetin</td>
<td>b</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>18618485</td>
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<tr>
<td>5. Receptor tyrosine-protein kinase erbB-2</td>
<td>ErbB2</td>
<td>P04626</td>
<td>2064</td>
<td>Quercetin</td>
<td>b</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>18618485</td>
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<tr>
<td>6. 5-lipoxygenase</td>
<td>ALOX5</td>
<td>P09917</td>
<td>240</td>
<td>Quercetin</td>
<td>i</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>15864785</td>
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<td>7. Estrogen receptor α</td>
<td>ESR1</td>
<td>P03772</td>
<td>2099</td>
<td>Kaempferol</td>
<td>a</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em></td>
<td>15182386</td>
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<td>8. Estrogen receptor β</td>
<td>ESR2</td>
<td>Q29271</td>
<td>2100</td>
<td>Quercetin; Kaempferol</td>
<td>a; i</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>15182386; 15182386</td>
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<tr>
<td>9. Tumor necrosis factor</td>
<td>TNFA</td>
<td>P01375</td>
<td>7124</td>
<td>Kaempferol</td>
<td>i</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em></td>
<td>17278014</td>
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<tr>
<td>10. Poly[ADP-ribose] polymerase I</td>
<td>PARP-I</td>
<td>P09874</td>
<td>142</td>
<td>Quercetin</td>
<td>i</td>
<td>Successful target (Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>17884996</td>
</tr>
<tr>
<td>11. Peroxisome proliferator-activated receptor gamma</td>
<td>PPARG</td>
<td>P3723</td>
<td>5468</td>
<td>Quercetin; Kaempferol</td>
<td>a; a</td>
<td>Successful target (Diabetes, Cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>18262572; 18262572</td>
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<tr>
<td>12. Aldose reductase</td>
<td>AR</td>
<td>P15121</td>
<td>231</td>
<td>Quercetin</td>
<td>i</td>
<td>Successful target (Diabetes)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>16806328</td>
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<tr>
<td>13. Calmodulin-dependent calcineurin A subunit α isofrom</td>
<td>CNA</td>
<td>Q08209</td>
<td>5530</td>
<td>Kaempferol</td>
<td>i</td>
<td>Successful target (Diabetes)</td>
<td><em>Ginkgo biloba</em></td>
<td>18506853</td>
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<tr>
<td>14. Nitric oxide synthase, inducible</td>
<td>NOS</td>
<td>P35228</td>
<td>4843</td>
<td>Kaempferol</td>
<td>i</td>
<td>Successful target (Ischemia reperfusion injuries)</td>
<td><em>Ginkgo biloba</em></td>
<td>17278014</td>
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<td>15. DNA topoisomerase 2-α</td>
<td>TOP2A</td>
<td>P43888</td>
<td>7153</td>
<td>Quercetin</td>
<td>i</td>
<td>Clinical trial target (cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
<td>16950806</td>
</tr>
<tr>
<td>16. Apoptosis regulator Bcl-2</td>
<td>Bcl2</td>
<td>P0415</td>
<td>596</td>
<td>Kaempferol</td>
<td>a</td>
<td>Clinical trial target (cancer and inflammation)</td>
<td><em>Ginkgo biloba</em></td>
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<tr>
<td>17. Apoptosis regulator BAX</td>
<td>Bax</td>
<td>Q07812</td>
<td>581</td>
<td>Kaempferol</td>
<td>i</td>
<td>Clinical trial target (cancer and inflammation)</td>
<td><em>Ginkgo biloba</em></td>
<td>15857606</td>
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(continued)
<table>
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<tr>
<th>Target name (abbreviation)</th>
<th>Uniprot.ID</th>
<th>Gene.ID</th>
<th>Compound</th>
<th>Interacting type</th>
<th>State of therapeutic target</th>
<th>Herb</th>
<th>PubMed.ID</th>
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<tr>
<td>18. Transcription factor AP-1</td>
<td>API</td>
<td>P05412</td>
<td>3725</td>
<td>(β)-Sitosterol</td>
<td>i</td>
<td>Clinical trial target (cancer and inflammation)</td>
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<tr>
<td>19. Cyclooxygenase-2</td>
<td>COX2</td>
<td>P33354</td>
<td>5743</td>
<td>Ursolic acid</td>
<td>i</td>
<td>Research target (cancer and inflammation)</td>
<td><em>Salvia officinalis</em></td>
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<tr>
<td>20. Caspase-9</td>
<td>Casp9</td>
<td>P52211</td>
<td>842</td>
<td>Gallic acid</td>
<td>i</td>
<td>Research target (cancer and inflammation)</td>
<td><em>Melissa officinalis</em></td>
</tr>
<tr>
<td>21. Interleukin-4</td>
<td>IL4</td>
<td>P05112</td>
<td>3565</td>
<td>Apigenin</td>
<td>i</td>
<td>Research target (cancer and inflammation)</td>
<td><em>Salvia officinalis</em></td>
</tr>
<tr>
<td>22. Steroid and xenobiotic receptor</td>
<td>SXR</td>
<td>O75469</td>
<td>8856</td>
<td>Ginkgolide a; Ginkgolide b</td>
<td>a; a</td>
<td>Research target (cancer and inflammation)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
</tr>
<tr>
<td>23. Caspase-3</td>
<td>Casp3</td>
<td>P42574</td>
<td>836</td>
<td>Gallic acid</td>
<td>i</td>
<td>Research target (neurodegenerative disease)</td>
<td><em>Melissa officinalis</em></td>
</tr>
<tr>
<td>24. Mitogen-activated proteins kinase</td>
<td>MAPK</td>
<td>P28482</td>
<td>5594</td>
<td>Quercetin; Gallic acid</td>
<td>b; b</td>
<td>Research target (neurodegenerative disease)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
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<tr>
<td>25. Sarcoplasmic/endoplasmic reticulum calcium ATPase 1/2</td>
<td>ATP2A1/2</td>
<td>O14983; P16615</td>
<td>487, 488</td>
<td>Quercetin</td>
<td>i</td>
<td>Research target (Diabetes)</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
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<tr>
<td>26. Cytochrome P450 1A2</td>
<td>CYP1A2</td>
<td>P05177</td>
<td>1544</td>
<td>Quercetin; Kaempferol</td>
<td>i; i</td>
<td>None</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
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<tr>
<td>27. Cytochrome P450 1A1</td>
<td>CYP1A1</td>
<td>P04798</td>
<td>1543</td>
<td>Quercetin; Kaempferol</td>
<td>i; i</td>
<td>None</td>
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<tr>
<td>28. Protein kinase C</td>
<td>PKC-B</td>
<td>P05771</td>
<td>5579</td>
<td>Quercetin</td>
<td>b</td>
<td>None</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
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<tr>
<td>29. Glutathione S-transferase P</td>
<td>GSTP1</td>
<td>P09211</td>
<td>2950</td>
<td>Quercetin</td>
<td>i</td>
<td>None</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
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<td>30. Nuclear factor NF-κ-B p65 subunit</td>
<td>RELA</td>
<td>Q04206</td>
<td>5970</td>
<td>Kaempferol</td>
<td>i</td>
<td>None</td>
<td><em>Ginkgo biloba</em>; <em>Melissa officinalis</em></td>
</tr>
<tr>
<td>31. Matrix metalloproteinase-1</td>
<td>MMP1</td>
<td>P03956</td>
<td>4312</td>
<td>Kaempferol</td>
<td>i</td>
<td>None</td>
<td><em>Ginkgo biloba</em></td>
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<td>32. Collagen α-(III) chain</td>
<td>COL3A1</td>
<td>P02461</td>
<td>1281</td>
<td>Ginkgolide b</td>
<td>b; b</td>
<td>None</td>
<td><em>Ginkgo biloba</em></td>
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<td>33. Platelet basic protein</td>
<td>PBP</td>
<td>P02775</td>
<td>5473</td>
<td>Ginkgolide b</td>
<td>i</td>
<td>None</td>
<td><em>Ginkgo biloba</em></td>
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<td>34. Steroid 17-α-hydroxylase/17,20 lyase</td>
<td>CYP17</td>
<td>P05093</td>
<td>1586</td>
<td>Gallic acid</td>
<td>i</td>
<td>None</td>
<td><em>Melissa officinalis</em></td>
</tr>
</tbody>
</table>

Note: Interacting type: a-Active; i-Inhibit; b-Bind
association between the targets and AD might be implicated [48].

To perform the association analysis, a group of AD-related proteins were collected. These proteins include two parts, i.e. the successful therapeutic targets for FDA approved drugs, and the disease proteins of AD. By searching the TTD database, five successful therapeutic targets were collected with the key word ‘Alzheimer’s disease’: acetylcholinesterase (AChE), 5-hydroxytryptamine receptor 2A (5-HT2A), cholinesterase (BChE), 5-hydroxytryptamine receptor 1A (5-HT1A) and Glutamate [NMDA] receptor (NMDAR). In addition, four AD disease genes were further retrieved from the Online Mendelian Inheritance in Man (OMIM) database [49]: amyloid precursor protein (APP), presenilin 1 (PSEN1), presenilin 2 (PSEN2) and Apolipoprotein E (APOE). Their corresponding proteins were obtained according to Uniprot database. Totally, nine AD-related proteins were curated.

Our association analysis was performed in the following procedures:

Firstly, PPI data from the HPRD [50], Mint [51], Intact [52], BioGRID [53], DIP [54] and MIPS [55] Database was downloaded to construct a comprehensive background network. Secondly, the nine ‘AD-related proteins’ were mapped onto the background network as well as the 34 target proteins. Then, to explore whether the herbal targets might relate to the ‘AD-related proteins’ at a higher level of organization, the shortest path length between both sets of proteins in the network was measured [48]. The final averaged shortest path length between the two groups of proteins was 3.118. To test whether the result is statistically significant, with ‘AD-related proteins’ fixed to the background network, 34 proteins were randomly taken from the background PPI network (10,523 proteins) as a control rather than the 34 herbal targets and nine ‘AD-related proteins’. After 10,000 times of randomization, the distance of each was obtained similarly. The statistical significance between the actual distance and those of random counterparts was estimated with the commonly used Z-score [56]. If the absolute value of Z-score is >3, the deviation between the actual value and the random ones is considered to be of significance. Despite of the difference of less-than-one on average, the 34 herbal targets exhibit highly significantly close network distance (3.118) to the nine ‘AD-related proteins’ (Z-score = 5.803), comparing to the randomly-selected 34 nodes from the background PPI network (3.677). We believe that with more knowledge accumulated, closer functional linkage of the herbal targets and AD might be inferred in future.

Second, the relationship between individual target protein and AD was further investigated by database and literature searching.

Overlapping between herbal targets and known therapeutic targets were identified with the information from TTD database and DrugBank database [57]. Among the 34 herbal targets, 3 are successful anti-AD targets, 11 are successful targets of five diseases, and another 11 are clinical trial targets or research targets of the same five diseases.

The three anti-AD targets covered 60% of the five known successful anti-AD targets as mentioned above. They are Acetylcholinesterase (AChE), Cholinesterase (BChE), and Glutamate [NMDA] receptor (NMDAR). Through inhibiting AChE and BChE, FDA-approved drugs [58], such as donepezil, galantamine and rivastigmine have been considered to be the first line pharmacotherapy for mild to moderate AD. Meanwhile, memantine has been the only approved drug for the treatment of moderate to severe AD by repressing NMDAR [59]. From this point of view, the anti-AD effect of the collected herbal ingredients, Huperzine A and Huperzine B, is probably contributed by inhibition of these successful therapeutic targets of AD.

In addition to the successful anti-AD targets, 11 targets have been recorded in TTD as successful targets of other diseases, such as various neurodegenerative diseases (e.g. Parkinson), ischemia reperfusion injuries, cancer, inflammation and diabetes. Specifically, serine/threonine–protein phosphatase 2B catalytic subunit α isoform (CNA) has been a successful target for Parkinson’s disease, which is shown among the 11 targets. Selective inhibition of inducible NO synthase (iNOS) was shown to be able to reduce ischemia reperfusion injuries [60], while iNOS was suggested to play an important role in Aβ-mediated damage [61]. It is reasonable to detect the relation between the anti-AD herbal targets and other neurodegenerative diseases as well as ischemia reperfusion injuries, because these are related to brain diseases and their therapeutic approaches are generally aiming at neuroprotection. Such kind of association has also been reported in previous studies [60, 62].

Interestingly, targets of other diseases: inflammation, cancer, and diabetes were retrieved as well in
our study. First, eight of the remaining nine targets have been reported as successful therapeutic targets for cancer and inflammation. Among them, Estrogen receptor 2 (ESR2) and Peroxisome proliferator-activated receptor gamma (PPARG) can both be hit by two herbal ingredients, while the other six are all hit by one individual ingredient, respectively, as indicated in Table 2. Literature search indicated their potential association with the process of AD. For example, ALOX5 was observed to be upregulated with aging [63, 64], while aging is one import factor for AD. Meanwhile, ESR1, TNFA and PARP-1 were suspected to be associated with AD in several studies [65–67]. Second, aldose reductase (AR) and PPARG are successful targets for Diabetes.

Although close correlation between the herbal targets and AD was not inferred by the statistical tests, there were many evidences suggesting their inner relationship. In summary, the herbal ingredients of collected anti-AD herbs can interact with a wide range of protein targets, where the majority of them (74%) are either successful or research targets for AD-related diseases, including the three successful therapeutic targets against AD. Inflammation is secondary to protein accumulation in ADs, while all the 16 therapeutic targets (including research and clinical) for inflammation in our list are also overlapping cancer targets. Three targets for diabetes, three targets for Parkinson and one for ischemia reperfusion injuries were also found within our anti-AD target list. These results imply that anti-AD herbs are primarily interacting with tens of important proteins critical to disease treatment. Three targets for diabetes, three targets for Parkinson and one for ischemia reperfusion injuries were also found within our anti-AD target list. These results imply that anti-AD herbs are primarily interacting with tens of important proteins critical to disease treatment. Although the relationship between these diseases is not fully understood, these targets are serving as the material basis for the therapeutic effects of the anti-AD herbs, as well as suggesting important cross-talks between AD and the other complex diseases.

**Part-two: target network analysis of molecular mechanism for anti-AD herbs**

The main purpose of this part is to answer the following questions: What pathways are herbal medicines involving in disease treatment? What is the mechanistic difference between herbal medicines and western drugs?

To better elaborate the holistic modulation of the anti-AD herbal medicines, an integrated ‘AD-related pathway’ was compiled based on the ‘Alzheimer’s disease pathway’ from KEGG Pathway database [68, 69] and human PPI data from online databases [50–55]. First, proteins involved in the ‘Alzheimer’s disease pathway’ were used as the seeds to obtain their partner proteins in the context of PPIs. Second, the direct interacting partners were used as a new query to fish out the secondary partners. The pathway was expanded step by step as more herbal targets could be included. Third, closely connected proteins were grouped together, and they were organized according to current knowledge of AD pathology. Fourth, in order to clearly display the underlying mechanism of the herbal medicines, the intermediate interacting partners were removed. Finally, the pathway was synthesized by ‘Pathway Diagrammer’ [70]. Of 34 target proteins, 28 can be mapped or connected to the pathway. As shown in Figure 2, several AD-related processes were involved, including proliferation, Aβ degradation and cell death. The targets can be organized into the following pathways: AD symptoms-associated pathways, inflammation-associated pathways, cancer-associated pathways, diabetes mellitus-associated pathways, Ca2+-associated pathways and cell proliferation pathways.

**Inhibiting the AD symptoms-associated pathways**

As being mentioned above, three successful therapeutic targets for AD can be regulated by anti-AD herbal ingredients, including Two GPCR family members (acetylcholinesterase and cholinesterase), and Glutamate [NMDA] receptor (NMDAR). These three receptors are all located at cell membrane, as being shown in Figure 2. The acetylcholinesterase and the cholinesterase are involved in the cholinergic system which has been thought to have direct influence on cognitive processes [71]. NMDAR is a glutamate-gated ion channel which is linked to the induction of excitotoxic neuron death [72]. It is pleased to see that the ingredients of *Huperzia serrata*, could inhibit acetylcholinesterase and cholinesterase [73], as well as block NMDA ion channels [74]. As the FDA approved anti-AD drugs usually improve symptoms of AD patients through inhibiting these targets, similarly, the anti-AD effects of herbs could probably be contributed by cognition enhancement or glutamatergic excitotoxicity reduction to prevent patients from memory and recognition deterioration.
Suppressing the inflammation-associated pathways

As described in Figure 2, a series of inflammatory factors could be inhibited by herbal ingredients. Specifically, kaempferol contained in Ginkgo biloba was shown with a reduction of TNF, NF-kB and iNOS protein levels in the aged gingival tissues [75], while another component of Ginkgo biloba, Quercetin could inhibit LOX5 [76]. Moreover, two ingredients of Salvia officinalis, Ursolic acid and Apigenin, are correlated with the suppression of COX-2 expression and the inhibition of IL4 synthesis [77, 78], respectively. Actually, evidence for the involvement of inflammatory process in AD pathogenesis has been documented for a long time [79], and higher peripheral concentrations of several cytokines, chemokines and nitrogen species have been observed in AD [80]. The downregulation of the inflammatory cytokines by herbal ingredients will probably help slowing down of AD progress.

Interfering with the cancer-associated pathways

Cancer-associated pathways could be modulated by the herbal ingredients, as displayed in Figure 2. Kaempferol was observed to decrease Bax level [81] and increase Bcl-2 activity [82] in human tumor cells, as well as inhibit MMP1 induction in 12-O-tetradecanoylphorbol 13-acetate-treated human dermal fibroblasts [83]. Quercetin was found to reduce PARP1 level in pulmonary epithelial cells [84], and inhibit GSTP1 in malignancies [85]. A component of Melissa officinalis named Gallic acid, was shown with decreased activity on Casp3 and Casp9 in 3T3-L1 pre-adipocytes [86], while β-Sitosterol contained in Salvia officinalis inhibited the expression of AP1 in HT116 human colon cancer cells [87]. As cancer has been found to be closely related to AD in a prospective longitudinal study [88], influencing the cancer-related machinery may constitute an important part of anti-AD effects of these herbal ingredients.

Regulating the Diabetes mellitus-associated pathways

As shown in Figure 2, Quercetin and Kaempferol served as agonists of peroxisome proliferator receptor gamma (PPARγ) as validated in the PPARγ reporter gene assay [89], while Quercetin could also inhibit AR activity by 54% as being measured in an enzyme assay [90]. In fact, PPARγ has been found to induce insulin signaling as was recently reported to contribute to modulating Aβ accumulation which has been recognized as a major causative factor in AD pathogenesis [91]. AR catalyzes the rate limiting step of the polyol pathway of glucose metabolism, and is thought to be involved in the pathogenesis of AD.
secondary diabetic complications [92]. Besides the well-known involvement in glucose metabolism, AR has recently been suggested by various groups to play a pivotal role in inflammatory pathologies which is tightly related to AD as mentioned above [93]. These results show that PPARγ activation and AR inhibition of the herbs might contribute to the anti-AD efficacy other than preventing diabetes role.

**Downregulating the intracellular Ca2+ homeostasis pathways**

Four Ca2+-related proteins were targeted by anti-AD herbal ingredients, such as Kaempferol and Quercetin, being represented as blue squares in Figure 2. Changes of intracellular Ca2+ are believed to accompany with almost the whole-brain pathology process that has been observed in AD. These processes include Aβ production, tau phosphorylation, mitrochondrial dysfunction and presenilins mutation [94]. This indicates that affecting Ca2+ modulation effects may play an important role in the anti-AD effects of the herbal ingredients.

**Participating in cell proliferation process**

Some proteins involved in cell proliferation were also modulated by the herbal ingredients, as marked in Figure 2. Ginkgolide a and Ginkgolide b have been reported to activate SXR in human primary hepatocytes [95], while Quercetin and Kaempferol could activate ESR2 and ESR1 respectively in primary rat neuronal cells [96]. Although the exact mechanism is not very clear, the regulation of cell proliferation might also be important for the herbal ingredients in the treatment of AD.

Overall, the above analysis on the ‘AD-related pathway’ suggests several possible anti-AD mechanisms for the herbal ingredients. The herbal ingredients could produce their anti-AD effect not only by improving the symptom of AD patients, but also by targeting the fundamental of the disease pathophysiology such as the inflammation-associated pathways, cancer-associated pathways, diabetes-associated pathways and so on.

As a complex network disease [97], accumulating evidences have keep indicating the association between AD and other pathological diseases in the past few years [79, 98, 99], although the underlying genetic and molecular connections still need further investigation.

In summary, from the target network perspective of these herbal ingredients, it is suggested that *Huperzia serrata* might produce anti-AD effects mainly through a symptom-improving way, while targets of *Salvia officinalis* are mainly involved in inflammation and cancer-associated pathways. As for *Ginkgo biloba* and *Melissa officinalis*, their targets are more diversely distributed in these fundamental pathways of AD indicating their more extensive anti-AD effects.

**CONCLUSION AND PERSPECTIVE**

Target proteins of anti-AD herbal ingredients were systematically collected and analyzed from a network perspective. Although the anti-AD herbs might relate to other therapeutic effects or diseases, their target proteins were found to be closely linked to AD. Some of the target proteins are successful therapeutic targets for AD or cross-talk diseases to AD (41.18%), while some others are clinical or research targets for these diseases (32.35%). Furthermore, the herbal targets were mapped to the ‘AD-related pathway’ to explore the underlying mechanism of the anti-AD herbs from the perspective of network pharmacology. As been observed, the herbs might treat AD symptomatically as FDA-approved anti-AD drugs do, or modify the disease through inflammation-associated pathways, cancer-associated pathways, diabetes-associated pathways, etc., which are closely related to AD.

In summary, our work may be beneficial to the anti-AD field in two aspects:

**The first aspect**

The underlying mechanism derived from ‘multiple-ingredients multiple-targets’ strategy of the anti-AD herbs might facilitate the development of new anti-AD therapy as a complementary to the current FDA-approved drugs. Besides the ‘multiple-ingredients multiple-targets’, other properties may also contribute to the therapeutic effects of anti-AD herbal medicines. Our comprehensive literature searching found that 7 of the 10 collected compounds could pass through the blood–brain barrier (BBB) to produce anti-AD effects. For example, quercetin and kaempferol have been shown to transport across the BBB in rats [100]. This information was also shown in Table 2.

On the other hand, although being viewed as low toxicity, the safety of the herbal medicines should also be noticed as adverse effects may lead to their failure in anti-AD drug discovery, development and marketing. Since few clinical and toxicological
studies have been performed to study the side effects of herbal medicines, databases and computational tools designed for studying drug side effects would be extremely useful. The recent developed side effect resource (SIDER) database [101] and Drug Adverse Reaction Database (DART) [102] has documented the relationship between drugs, ADR targets and known side effects. Additionally, several computational methods may also be helpful in the field of safety evaluation [103–106]. Of course, adverse effects would be closely related to dosage of the medicine while no bioinformatics report has been seen to incorporate the information. It is anticipated that above bioinformatics resources and computational techniques would be useful to future adverse effects calculation.

The second aspect
Recent studies on disease–disease association have indicated the cross-talks between different diseases. As shown by the human disease network (HDN) [107] and the expanded human disease network (eHDN) [108], the genetic origins for the majority of diseases are shared with other diseases, and most of them are linked to only a few diseases, while a small part of them are related to many different diseases. In our paper, the AD-related target network has provided a more systematic and clear decipher of AD pathogenesis, including the potential cross-talks with inflammation, cancer, and diabetes. There are some reports that people with AD, Parkinson’s and Huntington’s diseases have a significantly low risk of most cancers [88] and the reverse correlations also hold true. The observation in this paper that the cancer-associated pathways are modulated by the anti-AD herbal ingredients gives reasonable explanation to that. Secondly, it is known that brain studies on AD have indicated clear evidence for an activation of inflammatory pathways and long-term use of anti-inflammatory drugs is linked with reduced risk to develop the disease [109]. Since the cause and effect relationships between them are still under investigation, the widely repression of inflammation pathway by anti-AD herbal medicine may provide hints to the future fine tuning of inflammation to delay, prevent, or treat AD.

Lots of molecules have been studied at different stages for their potential anti-AD effects. Among them, herbal medicines are one promising source with typical ‘multiple-ingredients multiple-targets’ property. Our work has focused on the holistic understanding of this ‘multiple-ingredients multiple-targets’ mechanism for anti-AD herbal medicines at a molecular level. Suggested from MOA of herbs, future anti-AD therapy might be considered from multi-intervening several pathways by systematic evaluation the different roles of combinational components.

Key Points
- Since the mechanism of herbal medicines is featured as multiple-ingredients and multiple-targets network properties, integrated bioinformatics application can provide valuable support to decode the MOA of herb medicines intervening complex diseases such as AD.
- Holistic target network analysis of the anti-AD herbs indicated not only part of the common mechanism shared between herbs and known FDA-approved drugs, but also the interesting cross-talks between AD and other diseases.
- The proposed bioinformatics method could have a great potential if applied systematically to the inference of new therapeutic uses for natural compounds.

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References


