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ORIGINAL ARTICLE

Bacopa monnieri supplementation has no effect on serum brain-derived neurotrophic factor levels but beneficially modulates nuclear factor kappa B and cyclic AMP response element-binding protein levels in healthy elderly subjects

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Abstract

Background and Aim: *Bacopa monnieri* is an Ayurvedic herb that has been used for multiple conditions, most notably to augment cognition, particularly memory and attention. Multiple mechanisms, including raising brain-derived neurotrophic factor (BDNF), have been proposed and investigated in animal models that require translational studies in humans.
Methods: Bacopa was administered in an open-labeled study to cognitively healthy controls over a 3-month period. Cognition and mood were assessed using the Montreal Cognitive Assessment (MoCA) and geriatric depression scale (GDS) at the baseline and 3-month visit. Laboratories were assessed for safety and serum levels of mature (mBDNF) and proBDNF were quantified. In a subset of subjects, intracellular signaling processes were assessed using western blot analysis.
Results: Bacopa was provided to 35 subjects and was well-tolerated except for 4 (11%) subjects who early terminated due to known, reversible, and gastrointestinal side effects (i.e., nausea, diarrhea). Over the 3 months, the GDS and the total MoCA did not significantly change; however, the delayed-recall subscale significantly improved (baseline: 3.8 ± 1.2, 3-months: 4.3 ± 0.9; P = 0.032). Serum mBDNF and proBDNF levels did not significantly change. Cyclic AMP response element-binding protein (CREB) phosphorylation significantly increased (P = 0.028) and p65 nuclear factor kappa B (NF-κB) phosphorylation significantly decreased (P = 0.030).
Conclusion: These results suggest that Bacopa may exert an anti-inflammatory effect through NF-κB and improve intracellular signaling processes associated with synaptogenesis (CREB). The future placebo-controlled studies are recommended.
Relevance for Patients: *B. monnieri* will require larger, blinded trials to better understand potential mechanisms, interactions, and utilization.

1. Introduction

Bacopa monnieri ("Bacopa") is an Ayurvedic herb that has been used for centuries for pain, depression, and memory enhancement [1,2]. It has gained popularity over the years given its availability, safety [3], and limited side effects [4]. Bacopa has multiple key active ingredients (bacoside A and B (saponins), flavonoids and others) with various proposed mechanisms of action relevant to memory and aging that require further exploration including anti-amyloidogenic, antioxidant, and anti-inflammatory actions [2,5,6].

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A few studies have supported Bacopa's ability to augment the memory of cognitively healthy subjects [7,6]. One of these studies included a population 65 and older (similar age to the current study) and showed tolerance and response to Bacopa over 12 weeks when compared to placebo [8] and another larger study where results have yet to be published [9]. Although these studies are encouraging, it is still necessary to further delineate potential biological underlying mechanisms of Bacopa in human subjects.

One such proposed mechanism of Bacopa incorporates the neurotrophin and brain-derived neurotrophic factor (BNDF), which has been shown to be instrumental to learning and memory [10]. Clinical data suggest that healthy behavior such as exercise increases serum BDNF [11,12] and stressful life events [11] and depression [13] are associated with the lower BDNF. Antidepressant treatments have been associated with a rise in BDNF in some studies [14]. Interestingly, animal models of depression have shown, Bacopa treatment increases BDNF [15,16] in the hippocampus and frontal cortex [17]. In addition, Bacopa has been associated with increased CREB phosphorylation [15,18,19], a key regulator of BDNF production, and increased BDNF in the hippocampus [19,20] as well as reduction of inflammatory cytokines [21,22]. These and other pre-clinical studies [18,23-26] support exploring Bacopa's potential to modify intracellular pathways and raise BDNF because of the limited availability of human data published measuring BDNF after Bacopa administration [27].

Given the observed beneficial effects of Bacopa on cognition and the neuroprotective mechanisms suggested in the literature, we were interested in further assessing these pathways in a population who were at risk of the future cognitive decline. We hypothesized Bacopa administration to cognitively healthy older adults over 3 months would raise serum BDNF, specifically the mature form (mBDNF), and modify intracellular pathways related to BDNF production (CREB phosphorylation) measured from peripheral samples. A secondary aim was to study the impact of Bacopa on cognition and mood.

2. Methods

2.1. Subjects

We recruited subjects through local advertising and our clinic to participate in an IRB (Institutional Review Board) approved protocol (NCT03974399). After providing consent, subjects disclosed medical history and current medications and supplements, completed the geriatric depression scale (GDS), and were administered the Montreal Cognitive Assessment (MoCA). Subjects also answered a question rating their memory concerns (1- "not at all" to 5- "very concerned") and the number of days per week on average they exercised (1–7 days). They completed the LEC-5 (life event checklist) which is a standardized method of gathering a person's exposure to stressful life events over a lifetime [28]. Routine clinical laboratory tests (hematology and chemistry) were collected as well as samples for later analysis (see below). Key inclusionary criteria included age of 60–78 years old with an education corrected score of 25 or above on the MoCA and a score of 9 or below on GDS. Key exclusionary criteria were unstable medical conditions up to the discretion of the Principal Investigator, baseline laboratories deemed significant, and FDA-approved medications for depression.

Subjects were instructed to orally self-administer their assigned study product with or without food with no dietary restrictions once a day in the morning. Subjects were contacted by phone at 1 week, 1 month, and 2 months to report any adverse events (AEs). Subjects returned to the study site for Visit 2 at the end of 12 weeks. The study staff interviewed subjects about any changes in the subject's health since the first visit and if there was any change in exercise activity or their LEC-5. Routine clinical laboratory tests were obtained and samples for repeat testing were collected.

2.2. Product description of Bacopa (CDRI08)

Although Bacopa is available in multiple formulations, we selected one (CDRI 08) that has a long-established use in published research [4]. This formulation is available in the United States as Acumen, sold by Klaire Labs. Typical dosing of two capsules a day contains 320 mg of Bacopa (*B. monnieri*) whole plant, dried extract (CDRI 08). The most common side effects previously reported include nausea, stomach pain, and diarrhea. Toxicology of Bacopa has been previously summarized [3].

2.3. Safety analysis

AEs included severity and relationship to study product. AEs were classified into standardized terminology from the verbatim description (Investigator term) according to the Medical Dictionary for Regulatory Activities (MedDRA version 12.1 or higher).

2.4. Testing

Trained staff administered MoCA and reviewed the GDS for completion. Initial visits lasted an hour or less and were scheduled at a time convenient for the subject, most often between 10:00 and 15:00. Memory assessment: The MoCA is a validated 30-point single-page cognitive screen that assesses temporal orientation, praxis, executive skills, language functions, and memory recall. This brief measure takes about 20 min to administer and alternate versions were provided at each visit to minimize the possible confounding variable of practice effects, which have been shown to be most pronounced on speed-based and memory tasks. The GDS is a standard screen for depression where a score of 10–19 suggests mild depression and >19 is severe [29]. The MoCA and GDS were reviewed by the principal investigator (APK).

2.5. Sample collection

Approximately 30 mL of blood was collected without a fasting requirement typically between 10:00 and 14:00 and processed per our laboratory's established standard operating procedures. Serum was kept at room temperature for 30 min after collection and then centrifuged and aliquoted into 500 µL tubes for storage at −70°C. Laboratory staff were blinded to any clinical data.

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2.6. Quantification of pro and mature BDNF in human serum

Pro-BDNF and mBDNF levels were measured by ELISAs using the human proBDNF and the mBDNF ELISAs from Aviscera-Bioscience (CA, USA) which show reproducible, sensitive, and selective detection of both forms of BDNF in human serum [30].

2.7. APOE and BDNF genotyping

To provide some genetic context, the well-characterized memory risk factor APOE (apolipoprotein E) and the commonly measured BDNF single nucleotide polymorphism (rs6265) were genotyped. Genomic DNA was extracted from 300 µL whole blood (stored frozen at -80°C) using the PureGene Kit (Gentra systems) according to the manufacturer's instructions. DNA was diluted with nuclease free water to 10 ng/µL for APOE genotyping analysis. The APOE (rs7412; rs429358) polymorphisms were genotyped using direct APOE kit (EzWay Direct APOE Genotyping Kit; Koma Biotechnology, Seoul, Korea), following manufacturer's instructions. Genotype-specific fragments were separated by electrophoresis in a 3% agarose gel mixture containing 2% agarose and 1% metaphor agarose, stained with ethidium bromide. APOE alleles were determined as ε2 (rs7412(T)/rs429358(T)), ε3 (rs7412(C)/rs429358(T), or ε4 (rs7412(C)/rs429358(C)). For the purposes of evaluating a potential deleterious effect of the APOE-ε4 allele, patients with ε2/ε2, ε2/ε3, and ε2/ε3 genotypes were grouped as APOE-ε4(−), and ε3/ε4 or ε4/ε4 genotypes were grouped as APOE-ε4(+).

The BDNF (rs6265) G→A substitution causing the Val66→Met66 amino-acid substitution was assayed by polymerase chain reaction (PCR) amplification using the forward 5′-ACTCTGGAGAGCGTGAATGG-3′ and reverse 5′-ACTACTGAGCATCACCCTGGA-3′ primers. The amplification conditions were as follow: Pre-denaturation at 95uC for 5 min, followed by 40 cycles consisting of denaturation at 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, and post-elongation at 72°C for 5 min, with a final maintenance step at 4°C. The PCR products was next digested at 37°C with the restriction enzyme PmII (Eco72I), followed by a 3.0% agarose gel electrophoresis. The A allele (Met66) was identified as an uncut band of 171 base pairs (bp) while the G allele (Val66) was constituted by two cut bands, 99 and 72 bp long. Similarly, for the purposes of evaluating a potential effect of the BDNF polymorphism, patients with Val/Met and Met/Met genotypes were grouped into "Met carriers" and compared to the Val/Val genotype.

2.8. Biochemical assessment of intracellular signaling

BDNF activity and associated intracellular signaling are important for learning and memory [10]. Mature BDNF binds to the tropomyosin-related kinase receptor Type B (TrkB), which initiates steps to stimulate neurite outgrowth which includes increasing phosphorylation of CREB at serine 133 (Ser133), while pro-BDNF appears to stimulate p75 neurotrophin receptor (p75NTR) resulting in the activation of Janus Kinase (JNK) and NF-κB which promotes neurodegeneration and inflammation. The quantification of TrkB and p75NTR signaling was quantified by western-blotting in white blood cell lysates obtained at baseline and after 12 weeks. White blood cells were isolated using the ACCUSPIN System-Histopaque-1077 following manufacturer's recommendations (Sigma-Aldrich, USA) using 4 mL of whole blood using EDTA as an anti-coagulant. White blood cell lysates were prepared by resuspending white blood cell pellets with 400 microliters of mammalian protein extraction reagent MPER (Thermoscientific, USA) containing a cocktail of protease and phosphatase inhibitors (Thermoscientific, USA) and stored at -80°C. P75NTR signaling was analyzed by quantifying the levels of p75NTR, JNK phosphorylation, and p65NF-κB phosphorylation by western-blot using the following antibodies: p75NTR (D4B3) XP® Rabbit mAb #8238 (Cell signaling, CA, USA), Phospho-JNK (Thr183/Tyr185) (G9) Mouse mAb which detects JNK dually phosphorylated at Thr183 and Tyr185 (Cell signaling, CA, USA), and Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb which detects NF-κB p65 only when phosphorylated at Ser536 (Cell signaling, CA, USA). TrkB signaling was analyzed by quantifying the levels of total TrkB, phosphorylated TrkB, phosphorylated AKT, GSK3B phosphorylated at serine 9, and phosphorylated CREB using the following antibodies: TrkB (80E3) Rabbit mAb #4603 (Cell Signaling, CA, USA), Phospho-TrkB (Tyr515) Polyclonal Antibody (Thermofisher Scientific, USA), Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060 (Cell Signaling, USA), Phospho-GSK-3β (Ser9) (D85E12) XP® Rabbit mAb #5558 (Cell Signaling, USA), and Phospho-CREB (Ser133) (87G3) Rabbit mAb #9198 (Cell Signaling, USA). Western-blots and hybridization with the different antibodies were performed as we previously described [31,32]. The chemiluminescent blots were imaged with the ChemiDoc MP imager (Bio-Rad, CA, USA). The Band Analysis tools of ImageLab software version 5.0 (Bio-Rad, CA, USA) were used to select and determine the background-subtracted density of the bands in all the blots. Actin was used as a reference protein and chemoluminescent signals were standardized to actin levels for all the proteins analyzed. On each western-blot, one sample obtained at visit 1 and one sample obtained at visit 2 from the same subject were loaded. Samples from visit 1 were used as an internal reference for each subject, while values of visit 2 were expressed relative to the chemoluminescent signal values obtained at visit 1.

2.9. Statistical analysis

Summaries for each variable including the sample size, mean, and standard deviation (SD) are reported. The hypothesis of a normal difference in the values was tested using the D'Agostino-Pearson omnibus test. Paired-samples t-tests were performed to assess changes in key measures. Wilcoxon signed-ranks tests were used to compare pre- and post-intracellular signaling data after normalizing to actin and baseline visit. All analyses were performed with IBM SPSS Statistics Version 28, and *P*-values for comparisons lower than 0.05 were considered significant.

3. Results

3.1. Subjects

Forty-four subjects were screened for the study and nine were excluded (seven with low MoCA, one with elevated GDS, and one disclosed antidepressant use at screening visit) leaving 35 to

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receive Bacopa. Of the 35 subjects, 4 (11%) terminated early due to gastrointestinal side effects (a known side effect of Bacopa, and most within the first few weeks and all resolved after stopping study product), two were lost to follow-up, and one exited the study due to another unrelated diagnosis. The remaining 28 subjects were used for analysis.

The 28 subjects included 21 (75%) females and 7 (25%) males with a mean age of 70.1 ± 5.6 years. Most (96%) were non-Hispanic whites with one Asian. Table 1 summarizes these and other baseline characteristics.

3.2. Medical history

Several relevant medical conditions reported by the subjects included hyperlipidemia (54%), hypertension (32%), history of cancer (14%), diabetes (7%), and cardiac stent or bypass (4%). No subjects reported a history of atrial fibrillation, stroke, or carotid stenosis.

3.3. Assessment of Serum BDNF

One of the objectives of the study was to determine if serum levels of mBDNF or proBDNF changed after 3 months of Bacopa administration. As shown below in Table 2, no significant changes in these levels occurred.

3.4. Assessment of cognition and mood

Additional objectives of the study were to assess changes in cognition and mood after 3 months of Bacopa administration. As shown in Table 2, total MoCA scores and GDS did not change significantly; however, the total recall sub-score of the MoCA did significantly improve ($P = 0.032$).

3.5. Intracellular cell signaling from WBC's lysate

We had viable white blood cell lysates for 22 subjects to assess intracellular signaling. Using western blots, TrkB and p75NTR as well as JNK, p65NF-κB (referred to as "NF-κB"), AKT, CREB, and GSK3β phosphorylation levels in white blood cells were quantified relative to actin. Representative western-blots are shown in Figure 1. TrkB was not detectable and only a few subjects had detectable levels of JNK and p75NTR; therefore, these were excluded from the analysis. The remaining levels were assessed relative to baseline and NF-κB phosphorylation showed a significant decrease ($P = 0.030$), while CREB phosphorylation showed a significant increase ($P = 0.028$) and no significant changes were seen in AKT, or GSK3β phosphorylation as shown in Figure 2.

3.6. Self-report exercise, memory concert, and life events checklist (LEC)

No significant changes in self-reported exercise activity or memory concern occurred; however, 11 (39%) reported a change in their LEC. These included anything from the loss of a spouse to witnessing the pandemic on the news (five of the subjects had their second blood drawn during the early months of the COVID pandemic). The GDS remained stable over the two visits regardless of these changes in LEC-5 (Table S1).

Table 1. Baseline characteristics

Characteristics	N (%) or mean±SD
Total number	28
Females	21 (75)
Age (years)	70.1±5.6
Education: Bachelor's or higher degree	24 (86%)
BMI (kg/m^2)	24.6±4.4
Consume alcohol	24 (86)
Smoker (current)	1 (3.6)
Prior smoker	11 (39)
APOE4 carriers	5 (18)
Met carriers	10 (36)
Exercise days per week	4±2

BMI: Body mass index

Table 2. Average±SD for BDNF levels, MoCA scores and GDS score at each visit

Assessments	Baseline (pre)	Post (after 3 months of Bacopa)
mBDNF (ng/mL)	25.9±8.8	24.9±9.8
proBDNF (ng/mL)	11.1±24	9.7±22
MoCA (0 – 30)	27.3±1.7	27.8±1.7
MoCA recall (0 – 5)	3.8±1.2	4.3±0.9**
GDS (0 – 30)	2.5±2.5	± 3.2

Data for mBDNF and pro-BDNF limited to $n = 25$ where both blood samples were available. **$P = 0.032$ using paired t-test

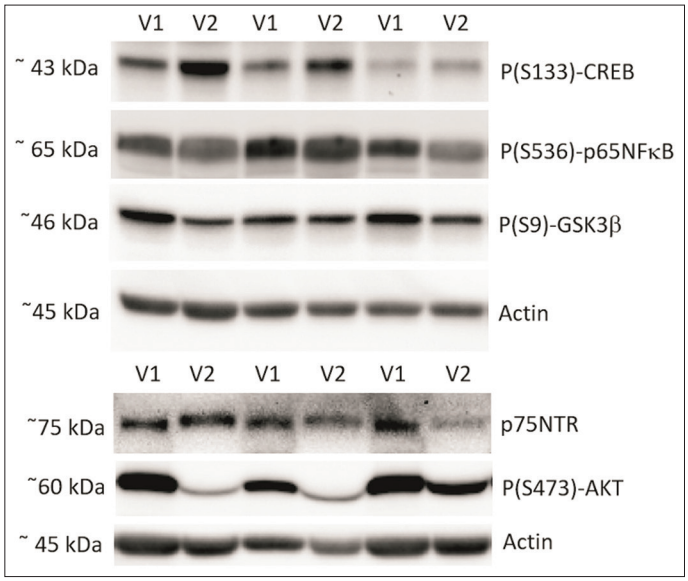

Figure 1. Representative western-blots of phosphorylation of each protein and the specific epitope (right) as well as the corresponding molecular weight (left) of visit 1 (V1) and visit 2 (V2).

3.7. Safety and AEs

Four subjects early terminated because of gastrointestinal side effects (diarrhea and nausea) that resolved shortly after stopping the study product. Of those that completed the study, seven subjects reported AEs and one was suspected to be related to Bacopa

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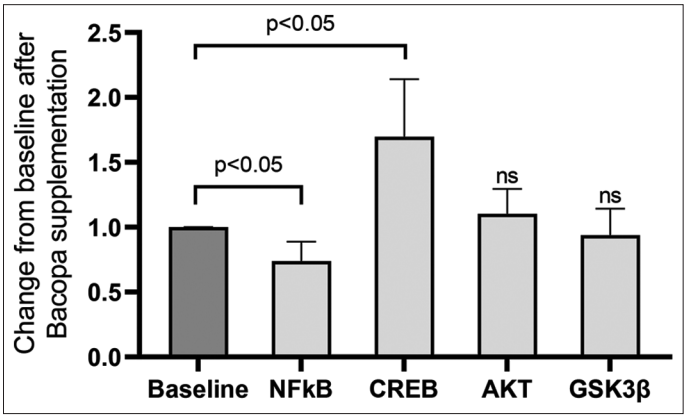

Figure 2. Bacopa was associated with a reduction in nuclear factor kappa B (NF-κB) and an increase in cyclic AMP response element-binding protein (CREB) phosphorylation. The histograms represent the ratio of the labeled intracellular signal/actin relative to the baseline level arbitrarily defined as 1 for each. Wilcoxon signed-rank test showed significant changes from baseline for NF-κB and CREB phosphorylation.

(fatigue), while others were suspected to be unrelated: back pain, rash that resolved, stomach flu, worsening arthritis, worsening cystitis, and tendon surgery. No SAEs (serious AEs) occurred. No clinically significant changes in basic labs (chemistries and complete blood count) were found.

4. Discussion

Bacopa has shown potential as an alternative method of augmenting cognition, albeit often in small non-blinded trials; however, the multiple proposed mechanisms of action support its continued examination. We were able to detect in a small and open-label study, subtle but biologically relevant changes that build on these pre-clinical mechanisms, most notably the increase in CREB phosphorylation, an important signaling molecule with multiple roles including supporting the production of BDNF. Interestingly, in Alzheimer's disease (AD), it has been proposed that amyloid interrupts CREB phosphorylation leading to reduced BDNF production [33]. We suggest the increase in CREB phosphorylation associated with Bacopa by one mechanism may translate into building cognitive reserve by providing support for BDNF production analogous to other healthy cognitive behaviors (e.g., exercise and stress reduction) associated with improved BDNF [11], but this will require further study.

Neurotrophic support is a recommended approach for AD prevention [34] and Bacopa is one of several nutraceuticals of interest with the potential to raise BDNF [35,36]. Although we did not detect a change in mBDNF or proBDNF, this may be partially explained by the micro-environment assessed (serum). Some report serum samples may be a reasonable reflection of central nervous system BDNF levels [37], while others suggest that plasma may be more accurate [38]. At present, the ability to measure BDNF in the human brain through imaging is not available; however, techniques under development in mice [39] may help better delineate the preferred sampling source.

Bacopa's association with increased CREB phosphorylation may have additional benefits in the inflammatory domain. Phosphorylated CREB has been proposed to directly inhibit NFκB activation by affecting the binding of CREB binding proteins (CBP/p300) to the NFκB complex, thereby inhibiting NFκB transcriptional activity and limiting pro-inflammatory responses [40]. These mechanisms may partially explain the reduction in NFκB phosphorylation reported here and some of the anti-inflammatory effects of Bacopa suggested by others [21]. Of note, we did not find significant changes in GSK3b, another regulator of NF-κB [41,42], or AKT, a potential modulator of GSK3b.

Following Bacopa supplementation, we found no change in the overall MoCA score but we did see a statistically significant improvement on the delayed recall suggesting a possible positive impact on memory. However, this finding, which represented less than one additional word recalled, may partially reflect a practice effect, and thus warrants further study over longer time spans. It is relevant to highlight that practice effects are typically highest for speed-based measures followed by delayed recall; therefore, a small contribution from Bacopa cannot be completely ruled out. Bacopa has been shown in numerous studies to improve or stabilize cognitive scores [6], most notably in those with attention deficit disorder, but also in those with cognitive impairment [2,43]. These results may be partially explained by alternative mechanisms including cholinesterase inhibition [2]. Donepezil, a commonly prescribed cholinesterase inhibitor indicated for AD, has been compared to Bacopa and found to be similar in benefit in a small but limited study of those with Mild Cognitive Impairment/AD [44,45]. We did not evaluate cholinesterase metabolites, but this could be assessed in the future clinical trials seeking better characterization of Bacopa's mechanisms of action [20].

Although late-onset depression has been associated with AD (see [46] for review), we limited this potential confounding variable by screening for depression and excluding those prescribed anti-depressants. As expected, we did not see any change in depression scores (GDS) over time as these averages remained in the normal range. However, some significant life stresses were reported on the LEC-5 without significant (albeit close in those with a change in LEC-5, Table S1) changes in the GDS. This may be partially explained by a lack of sensitivity of the GDS although interestingly, prior research has suggested Bacopa's potential in managing mood [16], and this may be an alternative explanation for these stable depression scores, which requires further study.

Safety data did not raise any new concerns with Bacopa, although this small study cannot supplant the importance of larger, double-blind, and placebo-controlled trials and the need to evaluate potential interactions. The study showed more evidence of gastrointestinal side effects which may be partially attributed to cholinesterase inhibition. Key limitations to the study include the small, limited diversity (96% white), predominantly female (75%), unblinded without placebo methodology with limited

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behavioral and cognitive testing, and a short follow-up period of 12 weeks, but this also allowed for a relatively straightforward study for the subjects to provide biological samples.

As we strive to develop additional methods to support cognitive health and reserve, components from long-used nutraceuticals such as Bacopa offer potential mechanisms that require continued investigation. The findings of increased CREB phosphorylation and reduced inflammation (reduction in NF-κB phosphorylation) associated with 3 months of Bacopa consumption along with a recent study's exploratory analysis suggesting favorable microstructural improvements after Bacopa supplementations [27] support further studies to better understand Bacopa's potential clinical effect.

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Conflicts of Interest

The authors declare no conflicts of interest.

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