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A Novel Neuroprotective and Antioxidative Efficacy of a Unique Combination of Standardized *Huperzia serrata*, *Convolvulus pluricaulis* and *Celastrus paniculatus* Extracts

Ahmad I[1], Swaroop A[2] and Bagchi D[*3]

[1]Cepham Life Sciences, Inc., Fulton, MD, USA
[2]Cepham Inc, Somerset, NJ, USA
[3]Pharmacological & Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, TX, USA

[*]**Corresponding author:** Bagchi D, Pharmacological & Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, TX, USA, E-mail: debasisbagchi@gmail.com

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Abstract

Physical and environmental stress in conjunction with hectic lifestyle and unhealthy food habits are the major cause of diverse neurodegenerative disorders. Oxidative neuronal injury and acetylcholine deficiency have a major impact on learning and memory retention. Most of the treatment strategies are based on the improvement of cholinergic function in the brain and one of the emerging therapeutic targets is to enhance the acetylcholine level in the brain. Standardized botanical extracts including *Huperzia serrata* (1% Huperzine A, CogniUp), *Convolvulus pluricaulis* (SP) and *Celastrus paniculatus* (JY) have been demonstrated to attenuate brain function by serving as a natural acetylcholinesterase (AChE) inhibitor. In this investigation, a unique combination of CogniUp, SP and JY (MZ001) was developed, which synergistically inhibited AChE and attenuated oxidative stress as reflected in reduced glutathione (GSH) values and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) inhibition. Concentration-dependent AChE inhibition kinetics was assessed individually using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg SP/mL, and 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg CogniUp/mL, respectively. It was observed that a combination of SP (12 mg/mL), JY (4 mg/mL) and CogniUp (0.125 µg/mL) provided the most efficacious and synergistic combination for the inhibition of AChE activity and oxidative stress. In another independent study, cultured Chinese Hamster Ovary (CHO) cells were pre-incubated with Cogniup (CP)(0, 0.0625, 0.125, 0.25 and 0.5 µg/mL) and a combination of SP, JY and Cogniup (12 mg/mL, 4 mg/mL and 0.125 µg/mL) (MZ001) followed by an oxidative exposure to 200 mM of H_2O_2 over the period 0-48 hours. Pretreatment of these cells with Cogniup or MZ001 prior to H_2O_2 exposure significantly elevated the cell survival, increased the levels of reduced glutathione. Similar protective efficacy was observed in DPPH assay. Overall, Cogniup and MZ001 exhibited dramatic neuroprotective efficacy against H_2O_2-induced oxidative damage, which may be important for clinical efficacy for the treatment of neuronal injury. Further studies are in progress to establish the therapeutic efficacy of MZ001 in neuroprotection.

Keywords: Huperzine A (CogniUp); *Convolvulus Pluricaulis; Celastrus Paniculatus;* Acetylcholinesterase (AChE); Reduced Glutathione (GSH); 1,1-diphenyl-2-picrylhydrazyl (DPPH); Cultured Chinese Hamster Ovary (CHO) cells; H_2O_2

Introduction

The human brain is one of the most vital, largest and complex tissue in the body, which is composed of 100 billion nerves which transmit signals and communicate in trillion of connections known as synapses and process a constant stream of sensory data [1]. Brain critically monitors body's actions, analyze the data and regulates the physico-chemical functions [2,3]. The brain stem regulates breathing, heart rate and other autonomic processes, the neocortex controls memory, learning and thinking, and cerebellum takes care of the body's balance, posture and coordination of movement [4-6].

However, hectic lifestyle, psychosocial and environmental stress, unhealthy food habits, and advancing age pose a potential threat on cognitive decline, memory impairment, anxiety, insomnia, learning and performance, attention-deficit disorder, short-term memory loss, depression, Parkinson's and Alzheimer's diseases [1]. Chronic stress alone has been reported to cause mental impairment/illness, kills brain cells, shrink the brain and modify brain structural pattern [7-9]. Similarly, the other cited elements such as unhealthy food habit or advancing age contributes to brain dysfunction and impairment [8-11]. The ideal therapeutic strategy is to boost the cholinergic function in the brain by enhancing the acetylcholinesterase (AChE) level in the brain vicinity [7-10].

Neuroprotective efficacy of a number of structurally diverse phytopharmaceuticals in medicinal plants including *Huperzia serrata, Bacopa monniera, Centella asiatica, Convolvulus pluricaulis, Celastrus paniculatus,* Panax ginseng, black pepper, turmeric and curcumin, trans-resveratrol, alpha-lipoic acid, Omega-3 and omega-6 polyunsaturated fatty acids, n-3/n-6 PUFAs, phosphatidyl serine and tocotrienols have demonstrated in boosting brain function, prevent cognitive decline, attenuate focus and attention [10-18]. Intake of seafood and fishes has been reported to preserve mental well-being, prevent or slow-down neurodegenerative disorders and promote cognitive capabilities [11,13].

Huperzine A, a sesquiterpene alkaloid naturally derived from *Huperzia serrata*, is an acetylcholinesterase (AChE) inhibitor, and N-methyl-D-aspartate receptor (or glutamate receptor) antagonist [19-22] has been demonstrated to improve cognitive performance in a broad range of animal models involving mice, rats, and monkeys with induced amnesia [23,24]. Huperzine A remarkably improved the retention of a learned task when tested 24 hours later in aged mice. Also, enhancement of learning and memory performance, increased retention, and faster retrieval [15,16].

During advancing age, loss of cholinergic neurons in the brain has been reported and this loss is instrumental in the process of memory impairment leading to dementia, while Huperzine A improves cholinergic function in the brain by inhibiting acetylcholine degradation [19-22,25-27]. Huperzine A has been demonstrated to prevent against myasthenia gravis (a deadly neuromuscular disorder) glaucoma and prevent nerve agent and pesticide toxicity [17,18].

Phytochemical analyses of standardized *Convolvulus plauricaulis* extract, locally known as Shankhapushpi (SP) demonstrated the presence of structurally diverse glycosides, coumarins, flavonoids, and alkaloids. SP works as nootropic by altering the availability of brain supply of neurochemicals (neutrotransmitters, enzymes, and hormones) by improving the brain's oxygen supply and optimizing blood flow to the brain, thereby increasing nutrient supply and stimulating nerve growth, which improves brain function, memory and focus [28-31].

Preclinical studies on various extract of SP reported significant improvement on learning behavior and memory enhancement, and hence, it is used as a brain tonic to promote intellect and memory and to alleviate nervous disorder and hypertension [24,28-31]. Clinical studies have demonstrated beneficial effects in patients with anxiety, neurosis and nervousness [24,28-31].

Standardized *Celastrus paniculatus* plant extract or seed oil (historically known as Jyotishmati or Malkangni)(JY), enriched in sesquiterpenes and beta-sitosterol, has long been known in Ayurveda to treat brain related disorders and dysfunctions, loss of mental ability and memory, improve memory and cognitive deficits [32-36]. JY also attenuated chronic stress-induced impairment of spatial learning and memory and chronic stress-enhanced anxiety-like behavior [33-37]. It induces a feeling of calm and peace; good sleep; and relief from anxiety, panic attack, physical stress and mental fatigue; and reduces the level of anxiety significantly [34-40].

The ideal therapeutic strategy is to improve the cholinergic function in the brain and the ideal treatment strategy is to boost the acetylcholine level in the brain without inducing potential toxicity. Based on the available literature, we assessed concentration-dependent neuroprotective efficacy of CogniUp, SP and JY, singly and in combination, against AChE inhibition, glutathione depletion and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) inhibition.

Materials and Methods

Test Materials

CogniUp (*Huperzia serrata* extract standardized to 1% Huperzine A) Batch number HUPA20180212, Shankhapushpi (standardized *Convolvulus plauricaulis* extract, SP) Batch number CP1810 and Jyotishmati (standardized *Celastrus paniculatus* extract, JY) Batch number CP1712, manufactured by Cepham (Somerset, NJ, USA), were used in this study.

Chemicals and Reagents

All reagents were of the highest purity available. Reduced Glutathione (GSH, also, Chemical Name: γ-L-Glutamyl-L-cysteinyl-glycine) CAS Registry Number 70-18-8 was procured from Cepham Inc., (Somerset, NJ). DPPH (2,2-Diphenyl-1-Picrylhydrazyl) CAS Registry Number 1898-66-4; Item No. 14805 procured from Cayman Chemical (Ann Arbor, MI, https://www.caymanchem.com/product/14805/dpph) and ascorbic acid CAS Registry Number 50-81-7; Item No. 14656 was procured from Cayman Chemical (Ann Arbor, MI, https://www.caymanchem.com/product/14656). Acetylcholinesterase (AChE) Assay Kit (Cat. No. 10674) from Cepham Life Sciences, Inc. (Fulton, MD, https://www.cephamls.com/acetylcholinesterase-assay-kit/). Appropriate solutions of all reagents were freshly prepared for estimation.

Protective Abilities of Shankhapushpi (SP), Jyotismati (JY) and CogniUp, alone and in Combination, in H_2O_2-Induced Acetylcholinesterase (AChE) Inhibition

AChE, present in the blood and at neuromuscular junctions and cholinergic synapses in the CNS, is involved in neural responsiveness and hydrolyzes the neurotransmitter acetylcholine to acetate and choline. Neuroprotective activity and AChE inhibitory effects of SP, JY and CP, alone and in combination were investigated in a dose-dependent manner (Table 1). Acetylcholinesterase (AChE) Assay Kit (Product No. 10674) manufactured by Cepham Life Sciences, Inc., based on modified Ellman's method [37] was used for measuring AChE activity in all the samples.

Dose-dependent AChE inhibition kinetics were assessed individually using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg SP/mL, and 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg CogniUp/mL (Table 1). Based on the observed results, we observed that a combination of SP, JY and CogniUp (12 mg/mL, 4 mg/mL and 0.125 µg/mL) provided the most efficacious and synergistic AChE inhibition (Table 1).

Standardized Botanical Extract	Concentrations of Standardized Botanical Extracts and Reference Standard (Ascorbic acid)	% DPPH Radical Inhibition
1% *Huperzia serrata* Extract (CogniUp)	0.125 µg/mL	28.2 ± 0.5
Convolvulus pluricaulis - (SP)	12.0 mg/mL	24.5 ± 2.1
Celastrus paniculatus- (JY)	4.0 mg/mL	16.2 ± 0.5
CogniUp + SP + JY	0.125 mg/mL + 12.0 mg/mL + 4.0 mg/mL	31.5 ± 0.3**
Ascorbic Acid	0 - 100 µg/mL (Reference Standard)	-

The % DPPH Radical Inhibition, showing antioxidant efficacy of different standardized botanical extracts of CogniUp, SP, JY and a combination of CogniUp + SP + JY was estimated using DPPH radical inhibition assay. **p < 0.001).
Table 1: % DPPH Radical Inhibition by Standardized *Huperzia serrata* (CogniUp, 1% Huperzine A), *Convolvulus pluricaulis* (SP) and *Celastrus paniculatus* (JY) Extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical

Protective effects of SP, JY and CogniUp, alone and in Combination, against H_2O_2-Induced Oxidative Stress and Reduced Glutathione (GSH) Depletion

Oxidative stress is implicated in the pathogenesis of diverse neurodegenerative disorders. We investigated the concentration-dependent antioxidant effect on reduced glutathione (GSH) content, using the method described by Ahmad and Suhail [38] and underlying molecular mechanism of selected botanical extracts CogniUp, SP and JY, singly and in combination, in cultured CHO cells. These cells were pretreated with CogniUp, SP and JY, singly and in combination, in different concentrations over a period of 2 hrs, then cells were washed with PBS, exposed to 600 µM Hydrogen peroxide (H_2O_2) over a period of 10 min. Cell viability and GSH levels were assessed. Cells were equally distributed in 96 well plates, after 24hrs of incubation the Cell viability was checked by trypan blue before and after treatment with SP, JY and CP. A standard curve for GSH was drawn and the OD was taken at 412 nm (Figure 1 and Table 2). Protection against GSH depletion was assessed individually using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg of SP/mL, and 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg of JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg of CogniUp/mL (Table 2). We observed that a combination of SP, JY and CogniUp (12 mg/mL, 4 mg/mL and 0.125 µg/mL) provided the most efficacious and synergistic protection against GSH depletion (Table 2).

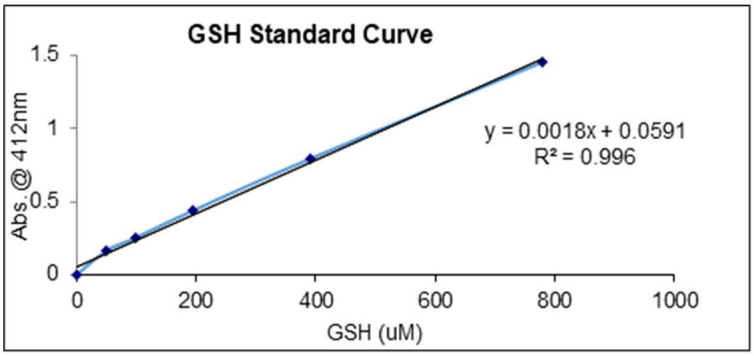

Figure 1: GSH Standard Curve (OD) was taken at 412 nm

Samples (Concentration)	Reduced (GSH) Content (µM)
Control Buffer	327 ± 18.6
H_20_2 (600 µM)	88.5 ± 7.5*
1% *Huperzia serrata* Extract (CogniUp) (0.125 µg/mL)	347 ± 18.7**
Convolvulus pluricaulis - (SP) (12 mg/mL)	328.9 ± 20.5**
Celastrus paniculatus - (JY) (4 mg/mL)	339.1 ± 31.2**
CogniUp (0.125 µg/mL) + SP (12 mg/mL) + JY (4 mg/mL)	337.5 ± 24.8**
CogniUp (0.125 µg/mL) + H_20_2 (600 µM)	308.3 ± 26.7**
SP + H_20_2 (600 µM)	250.5 ± 21.2**
JY + H_20_2 (600 µM)	284.4 ± 24.3**
CogniUp (0.125 µg/mL) + SP (12 mg/mL) + JY (4 mg/mL) + H_20_2 (600 µM)	328.5 ± 30.7**

GSH depletion of different standardized botanical extracts of CogniUp, SP, JY and a combination of CogniUp + SP + JY was estimated against H_2O_2-induced GSH depletion assay. *p < 0.05; **p < 0.001
Table 2: Comparative Protective Effects of SP, JY and CP Against GSH Depletion

Comparative DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Inhibition by SP, JY and CogniUp, Singly and in Combination

The effects of three standardized botanical extracts *Huperzia serrata* (CogniUp), *Convolvulus pluricaulis* (SP), and *Celastrus paniculatus* (JY), individually as well as in combination, on DPPH radical inhibition was estimated, using a modified method of Liyana-Pathirana and Shahidi and Okawa *et al.* [39,40]. A 5 mM solution of DPPH was prepared in methanol from the stock 100 mM solution. 200 µL of each herbal extract of CogniUP, SP, JY at previously indicated concentrations, individually, and in combination of CP (0.125 µg/mL) + SP (12 mg/mL) + JY (4 mg/mL) were added into a 96 well microwell plate. 5 mM DPPH (6 µL) was added into each well containing these herbal extracts, singly and in combination. Ascorbic acid was used as a reference antioxidant standard compound (IC_{50} 3 µg/mL). The microwell plate was kept in dark for 30 min at room temperature. Absorbance of each well was read at 517 nm.

The DPPH % Inhibition was calculated using the equation below:

DPPH % Inhibition = $[(Abs_{Control} - Abs_{Sample})/Abs_{Control}] \times 100$

Where $Abs_{Control}$ is the absorbance of DPPH = Methanol and Abs_{Sample} is the absorbance of DPPH + Herbal Extract or Standard.

Statistical Analysis

The results are expressed as means ±SD. All the data of different treated groups as compared to the control groups were statistically analyzed, using Student's t-test and values $p<0.05$ were considered significant.

Results

Acetylcholinesterase (AChE) Inhibition

Concentration-dependent AChE inhibition kinetics was assessed individually using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg SP/mL, and 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg CogniUp/mL (Table 1). Based on the results of these concentration-dependent data, we used a combination of SP, JY and CogniUp (12 mg/mL, 4 mg/mL and 0.125 µg/mL), which provided the most efficacious and synergistic AChE inhibition (Table 1).

Reduced Glutathione (GSH) Depletion

Concentration-dependent protective efficacy of SP, JY and CP were assessed, individually and in combination, against H_2O_2-induced GSH depletion. Several combinations were assessed using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg of SP/mL, and 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg of JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg of CogniUp/mL (Table 2). Based on the results of these concentration-dependent data, we used a combination of SP, JY and CogniUp (12 mg/mL, 4 mg/mL and 0.125 µg/mL), which provided the most efficacious and synergistic protection against GSH depletion (Table 2).

DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Inhibition by SP, JY and CogniUp, Singly and in Combination

Comparative DPPH radical inhibition was assessed using various concentrations of SP at 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg of SP/mL. While the same experiment was conducted using 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg concentrations of JY/mL, and 0, 0.0625, 0.125, 0.25 and 0.5 µg concentrations of CogniUp/mL. Similar results were observed in DPPH radical inhibition. The optimal combination of 12 mg/mL of SP, 4 mg/mL of JY and 0.125 µg/mL of CogniUp provided the maximal inhibition. (Table 3).

Samples (Concentration)	AChE Inhibition (%)
1% *Huperzia serrata* Extract (CogniUp) (0.125 µg/mL)	30.18%
Convolvulus pluricaulis - (SP) (12 mg/mL)	27.63%
Celastrus paniculatus- (JY) (4 mg/mL)	25.86%
CogniUp (0.125 µg/mL) + SP (12 mg/mL) + JY (4 mg/mL)	37.3%
CogniUp (0.25 µg/mL) + SP (12 mg/mL) + JY (4 mg/mL)	33.84%

Acetylcholinesterase (AChE) inhibition of different standardized botanical extracts of CogniUp, SP, JY and a combination of CogniUp + SP + JY was estimated
Table 3: Comparative Protective Effects of SP, JY and CP Against AChE Inhibition

Based on the experiments on these three critical components, we inferred that a combination of 12 mg/mL of SP, 4 mg/mL of JY and 0.125 µg/mL of CogniUp is a safe, novel synergistic formulation, which may demonstrate a significant efficacy in boosting neuronal health and antioxidant potential.

Discussion

It is important to emphasize that the number of people suffering from cognitive dysfunctions in United States in twice the population of New York city alone. In United States, more than 16 million people suffer from diverse cognitive disorders [Cognitive impairment: A call for action, for now. https://www.cdc.gov/aging/pdf/cognitive_impairment/cogimp_poilicy_final.pdf (accessed July 22, 2019)] [44]. Cognitive impairment has been reported especially in middle age, but mostly in aging population. Thus, cognitive impairment ranges from mild to severe. An array of mild cognitive impairment includes trouble remembering and learning new things, attention deficit disorders, focus, concentration, or making decisions, which creates problems in their daily life. It is worthwhile to mention that attention deficit disorder is quite common in children and young adults [50]. However, with these mild impairments, people are still able to perform their daily activities. But cognitive impairment jumps dramatically as soon as people cross the age of 65 years. It has been reported that about 5.1 million Americans aged 65 years or older may presently suffer from Alzheimer's disease, which is expected to rise to 13.2 million by 2050. Severe neurological impairment may lead to losing the ability to communicate, write, understand or determine the importance of some important matters, or survive independently [44].

Furthermore, increasing environmental pollutants, diesel exhaust, UV radiation and ozone layer depletion, heavy metal toxicity and hazardous chemical wastes are posing increasing threats in neuronal damage, stroke, brain ischemia and cancer [45,46].

It is very alarming to note that presently more than 10 million family members providing unpaid care to a person with a cognitive impairment suffering from diverse cognitive impairments including dementia, Parkinson's and Alzheimer's disease [44]. In a detailed report in 2009, about 12.5 billion hours of unpaid medical care were provided which was estimated to $144 billion. In addition, more in-home or institutional care and unpaid assistance by family and friends were provided to people suffering from Alzheimer's disease and other forms of cognitive impairment [44].

Several pharmaceutical therapeutics have been developed, which are very expensive and mostly associated with adverse side effects. A significant number of standardized botanical supplements, vitamins and micronutrients are increasingly becoming popular as brain healthy diet(s) and serve as natural neuroprotective agents, which prevents neuronal damage, cognitive oxidative stress and neurodegeneration, and thus delay the onset of cognitive decline [47-49]. Examples of standardized botanicals and other nutraceuticals include *Huperzia serrata, Convolvulus pluricaulis, Celastrus paniculatus, Curculigo orchioides, Ginko biloba, Curcuma longa, Centenella asiatica, Bacopa monniera, Panax ginseng,* lycopene, structurally diverse bioflavonoids, anthocyanins and polyphenols, different types of nuts including walnuts and almonds, black pepper, alpha lipoic acid, fish oil, tocotrienols, royal jelly, trace amounts of copper, zinc, selenium and manganese supplements [47]. In addition, consumption of marine fishes and general seafood has been used for long-term nutritional invention against mental health deterioration and slow the neurodegeneration process and preserve mental health and integrity [11,13]. However, water pollution with mercury, arsenic and chemical waste are posing significant threat of toxicity [48]. Careful measures and risks/benefits for each supplement must be done before choosing a nutraceutical supplement.

In this manuscript, we have short listed three standardized botanical extracts including standardized *Huperzia serrata, Convolvulus pluricaulis* and *Celastrus paniculatus* extracts and determined their efficacy, individually and in combination, against AChE inhibition, reduced glutathione (GSH) depletion and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) inhibition.

Huperzine A improves cognitive performance in many species of animals involving mice, rats, and monkeys with induced amnesia [23,24]. Furthermore, it improves performance in mice and rats in running through mazes, as well as protected young and aged animals against sodium nitrite, cycloheximide, carbon dioxide-treated, and electroconvulsive shock-induced passive response [23,24,49]. Huperzine A improves cognitive performance in a broad range of animal models involving mice, rats, and monkeys with induced amnesia, and remarkably attenuate the retention of a learned task when tested 24 hr later in aged mice [23,24,49]. Enhancement of learning and memory performance, increased retention, and faster retrieval processes were reported following supplementation of Huperzine A [23,24,49].

Research studies demonstrated that learning and memory retention following supplementation of Huperzine A was longer as compared to other existing AChE inhibitors [22,50]. AChE inhibitory efficacy of Huperzine A is 8-fold and 2-fold more as compared to donepezil and rivastigmine, respectively [51]. Loss of cholinergic neurons occurs during aging process. This loss is significantly detrimental in the acceleration of memory impairment leading to dementia. Mechanistically, Huperzine A potentially improves cholinergic performance and function by inhibiting acetylcholine degradation in the brain [21,25,50,52]. It targets the appropriate sites of AChE and prevents the selective degeneration of acetylcholine-producing neurons in the brain and enhances the availability of acetylcholine in the brain of subjects suffering from neurological disorders, muscle contraction, and dementia between nerves and muscle to function better and protects the neuronal cells. Improvement in neuromuscular cholinergic transmission leads to potential improvement in memory retention. Lin et al (1996) [53] demonstrated a unique pathophysiological mechanistic pathway of Huperzine A in the preservation of neurotransmitter acetylcholine, which carries electrical impulses from one nerve to another. In a normal brain function, AChE serves a housekeeping function by breaking down the acetylcholine. Acetylcholine breaks down into an acetate moiety and choline. The choline is then transmitted back into

the nerve ending to be used again to make acetylcholine. People with Alzheimer's disease demonstrate a potential deficiency of acetylcholine because of the damaged brain cells. Huperzine A, in turns, protects AChE from breaking down acetylcholine and, thereby, prevents the deficiency, and, in turn, improves brain functions [54-56].

The other ingredient used in this investigation is Convolvulus pluricaulis (Shankhapushpi, SP, known as morning glory), a perennial herb with branches spread on the ground and can be more than 30 cm long, which is Ayurvedic medicinal plant to boost "medhya" (meaning intelligence, memory enhancer, relieving tension, mental calmness and mental abilities) in India and Southeast Asia for approximately 5,000 years [30,31,57,58]. Shankhapushpi flowers are deep blue or purple in color, shaped like a "Sankha" (means conch, which is blown to make sound), while the leaves are elliptic in shape. This plant abundantly grows in India in sandy and stony areas, preferably in a dry climate [29-31,59]. The whole plant including flowers is used in Ayurveda to treat memory loss, long-term memory enhancement, insomnia, mental stimulation, and rejuvenation therapy [31,60]. SP also reported to reduce anxiety, neurosis, mental stress, work-related stress, and depression [61]. SP is nootropic, which is referred to as a brain tonic as well as memory and cognitive enhancer, which increases concentration and focus.

SP is enriched in glycosides, coumarins, flavonoids, and alkaloids including β-sitosterol, ceryl alcohol, hydroxycinnamic acid, octacosanol, tetracosane, scopoline, octacosanoltetracosane, scopoletin, convolvidine, subhirsine, convolvine, and phyllabine, along with glucose and sucrose, which are the key constituents of glycosides, as well as 20-oxodotriacontanol, tetratriacontanoic acid, and 29-oxodotriacontanol [31,62,63].

SP has been demonstrated to boost neuronal health by modulating the neurochemistry and keeps brain cells healthy and functional. Research studies demonstrated its efficacy in ameliorating mental weakness, forgetfulness, memory loss, low retention power, anti-ageing, and diverse central nervous system (CNS) disorders, including insanity, epilepsy, and nervous debility [28,29]. Mechanistically, SP regulate the production of stress hormones including adrenaline and cortisol to reduce stress and anxiety [61,64-66]. It also serves as a tranquilizer and psychostimulant [66,67]. Demonstrated to prevent memory loss and decrease the blood cholesterol, triglycerides and phospholipid levels [28]. Consumption of SP has been exhibited to retard memory loss and improve memory in neurodegerative diseases such as Parkinson's syndrome and AD [28]. It is also very popular to treat insomnia effectively, and in improving memory function in diabetics [65].

The third botanical plant was Jyotishmati (*Celastrus paniculatus* Willd., JY), a woody scrambling or climbing shrub climbs up to over 10 meters, another proven Ayurvedic herb for "Tree of Life" and "Medhya Drug" (brain tonic) and diverse therapeutic functions, which grows at an altitude of 1800 m in the subtropical Himalayas. It is widely used by the tribal people especially in Orissa, Maharashtra, and Andaman and Nicobar Islands [68-70].

Ayurvedic application has been reported mostly for boosting brain function, intellect-promoting and overall cognitive health, as well as anti-nephrotoxic and anti-inflammatory efficacies.

A diverse number of beneficial phytochemicals including alkaloids, glycosides, phytosterols and sesquiterpene have been reported in JY. Some of the notable alkaloids include Celapanin, Celapanigin, Celapagin, Celastrine, Pristimerin and Paniculatine, as well as dipalmitoyl glycerol, a sesquiterpene [32,33,68,69].

The seed oil and fruit are commonly used for its tranquillizing, sedative, antioxidant and wound-healing activities [68-70]. The bark is abortifacient, depurative, and used as a brain tonic. The leaves are emmenagogue and the leaf sap is a good antidote for opium poisoning. The seeds are acrid, bitter, thermogenic, emollient, stimulant, intellect-promoting, digestive, laxative, emetic, expectorant, appetizer, aphrodisiac, cardiotonic, anti-inflammatory, diuretic, diaphoretic, febrifuge and tonic, and can treat abdominal disorders, leprosy, skin diseases, paralysis, asthma, leucoderma, cardiac debility, inflammation, nephropathy, amenorrhea, dysmenorrhea [32-35]. The leaves contain alkaloids, a glycoside and coloring matter, whereas the oil extracted from seeds contains sterols, alkaloids, and a bright coloring matter, Celapanin, Celapanigin, Celapagin, Celastrine and Paniculatine are some of the important alkaloids present in the seeds. Ayurvedic literature demonstrated the efficacy of JY in boosting mental clarity and intelligence [32-40].

In this study, we determined concentration-dependent neuroprotective efficacy of safety-affirmed CogniUp (*Huperzia serrata* extract standardized to 1% Huperzine A), SP (*Convolvulus pluricaulis*) and JY (*Celastrus paniculatus*), singly and in combination, against AChE inhibition, glutathione depletion and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) inhibition. We determined synergistic efficacy at several combinations of CogniUp, SP and JY, and finally discovered a novel synergistic combination of CogniUp, SP and JY (0.125 µg/mL, 12 mg/mL and 4 mg/mL). This novel antioxidant combination may serve as novel formulation for potential applications in memory enhancement, mental focus and clarity and intelligence, and related neurological disorders.

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