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Multifunctional neuroprotective effect of Withanone, a compound from *Withania somnifera* roots in alleviating cognitive dysfunction

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ABSTRACT

Alzheimer's disease (AD) is a chronic disorder that slowly worsens and impairs the person's memory, learning, reasoning, judgment, communication and familiar tasks with loss of orientation. AD is characterized clinically by cognitive deficit and pathologically by the deposition of β amyloid plaques, neurofibrillary tangles, associated with degeneration of the cholinergic forebrain. Withanone (WS-2), a compound isolated from root extract of *Withania somnifera* at doses administered orally/day to wistar rats for duration of 21 days showed significant improvement in the cognitive skill by inhibiting amyloid β-42 and attenuated the elevated levels of pro-inflammatory cytokines like TNF alpha, IL-1 beta, IL-6, MCP-1, Nitric oxide, lipid peroxidation and both β- and γ-secretase enzymatic activity. Administration of WS-2 also significantly reversed the decline in acetyl choline and Glutathione (GSH) activity. None of the treatments that are available today alter the underlying causes of this terminal disease. Few preliminary clinical treatments have demonstrated that some plant medicines do ameliorate and improve memory and learning in patients with mild-to-moderate AD. WS-2 showed promise in AD treatment because of cognitive benefits and more importantly, mechanisms of action with respect to the fundamental pathophysiology of the disease, not limited to the inhibition of AChE, but also include the modification of Aβ processing, protection against oxidative stress and anti-inflammatory effects.

1. Introduction

Alzheimer's disease (AD) is irreversible neurodegenerative disorder causing deterioration of brain function affecting about 20–30 million individual the world over [1]. This is an unremitting age-related, chronic disorder that destroys mental capacities and functions with an enormous unmet medical need, leading to progressive disturbances of cognitive functions including memory, [2] disorientation, aphasia, constructive difficulties, judgment and performance disorders. Characteristic neuropathological findings include extracellular neuritic plaques containing the β-amyloid peptide [3] and neurofibrillary tangles. Aβ protofibril activate microglia, inciting a pro- inflammatory response and release of neurotoxic cytokines [4]. The inflammatory response associated with the presence of neuritic plaque or Aβ accumulation is involved in the neuronal damage and progression of the disease [5] .

In traditional Indian systems of medicine, the Indian medicinal plants have been used in successful management of various disease conditions. In Ayurveda *Withania somnifera* (Ashwagandha) is considered a revered rasayana herb. Rasayana in early ayurvedic medicine means the science of lengthening lifespan. Different parts (leaves, stem, flower, root, seeds, bark and even whole plant) of *Withania somnifera* have been recommended as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia etc. [6]. The therapeutic use of Ashwaganda for brain related disorders like anxiety, cognitive and neurological disorders and Parkinson's disease is supported by clinical trials and pre-clinical research. Ashwaganda is also used therapeutically as an adaptogen in patients that suffer from nervous exhaustion, insomnia, stress, and as an immune stimulant in patients with low white blood cell counts in blood [7].

Plant derived agents have been tested in animal (*in vivo*) and cell line based models (*in vitro*) of AD and these have shown multi-functional properties that include pro-cholinergic, anti-oxidant, anti-amyloid and anti-inflammatory activities. *Withania somnifera* root extract reversed the scopolamine-induced disruption of acquisition and

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retention and attenuated the amnesia produced by acute treatment with electro convulsive shock [8]. In another study conducted, it was found that *Withania somnifera* extracts caused an increase in cortical muscarinic acetylcholine receptor capacity which might partly explain the cognition-enhancing and memory-improving effects of the extracts as observed in both animals and humans [9]. Despite extensive research on the restorative effects of *W. somnifera* extract in different pathological conditions, there are no studies that explored the possible role of its constituent compounds in the treatment of AD and to elucidate the mechanism of action of the most active compound.

The objective of this study was to analyze different constituent compounds present in the root extract of *W. somnifera* for its anti-Alzheimer's effect and zero in on the component that shows significant activity to be taken up for detailed pre-clinical investigations. This study aimed to evaluate a natural product with anti-Alzheimer's activity to establish the scientific rationale and mechanisms of action against AD. The association of AD with autoimmune and inflammatory diseases suggests a link between it and immunological dysregulation. Research in this field paves the way to discover new treatments for AD via immune-therapy.

Neuroinflammation plays an integral role in AD development and precede plaque and tangle formation leading to impairment in learning and memory [10]. Pro-inflammatory mediators, TNF-alpha, Interleukin -6 (IL-6), Interleukin-1beta (IL-1β) are secreted by microglia and astrocytes surrounding Aβ neuritic plaques [11,12]. A second general category of cytokine action is manifested by anti-inflammatory cytokines such as IL-1, IL-4 and IL-10. These inhibitory cytokines can suppress pro-inflammatory cytokine production and action. Upregulation of a number of chemokines, including monocyte chemotactic protein-1 (MCP-1), is associated with Alzheimer's disease (AD) induced pathological changes. [13]. Amyloid precursor protein (APP), and β- and γ-secretases are the principal mediators involved in Aβ production. Modulation or inhibition of β- and γ-secretases and/or activation of α-secretases by a plant moiety should be a promising lead for the treatment of AD.

Oxidative stress in the brain plays an important role in the pathophysiology of AD [14]. The brain is extremely vulnerable to oxidative stress, as concomitant low activity and capacity of antioxidant protection systems allow for increased exposure of target molecules to free radicals.

In this study the role of CNS-infiltrating T cells and their related cytokine expressions was examined. Increased expression of microglial activation and Aβ deposition by the T cells impaired the cognitive function [15]. In addition, Th17 related autoimmune reaction plays an important role in the disease pathogenesis. Microarray analysis of peripheral blood mononuclear cells, shows that many Th17 immunity related molecules are up-regulated after the onset of Alzheimer's disease.

These plant based AD treatments can have enormous consequences for further research in to the drug development efforts for AD and possibly other neurodegenerative conditions.

2. Materials and methods

2.1. Plant material

The roots of *W. somnifera* (AGB002) were collected from the experimental field of Indian Institute of Integrative Medicine-CSIR, Jammu. The variety is characteristic for its stability, disease resistance, strong adaptability, high leaf biomass and root yield [16] A voucher sample number 28912 is retained and deposited at Janaki Ammal Herbarium, IIIM, Jammu, J & K state, India. The material was ensured to be free from pathogens, aflatoxins, pesticidal residues and heavy metals to meet WHO guidelines of purity and safety [17].

2.2. Extraction of test material

Test material (1 kg) was ground to coarse powder. The powdered roots were percolated four times with ethanol: water (1:1) at room temperature. The combined extract was filtered, centrifuged and concentrated to 1/6th of the original volume under reduced pressure in a thin film evaporator at 50 ± 5 °C. The procedure resulted in 158 g of greenish yellow, hygroscopic extract of powdered material, which was stored in desiccating conditions .

2.3. Isolation of chemical constituents

For the isolation of withanolides/glucowithanolides, the aqueous ethanolic extract 50 g was dissolved in water 200 ml and the solution was successively extracted with chloroform and n-butanol in a separating funnel. Both chloroform and n-butanol fractions were separately concentrated under reduced pressure to yield the residues containing withanolides and glucowithanolides. The withanolides were monitored by TLC system $CHCl_3$: MeOH (19:1) and glucowithanolides by TLC system $CHCl_3$: MeOH (4:1). The isolation and purification of withanolides/glucowithanolides [Withanolide A (WS-1), Withanone (WS-2), Withaferin A (WS-3), Withastramnolide (WSC), 27- Hydroxy withanone (WSCO) and Withanoside IV (WSG-3)] obtained from the chloroform and n-butanol extracts were carried out by a combination of different separation techniques column chromatography, preparative TLC, preparative HPLC and centrifugal adsorption chromatography (chromatotron) followed by crystallisation in appropriate solvents. Further, HPLC analysis of isolates WS-1, WS-2, WS-3, WSC, WSCO, WSG-3, WSG-P established the purity of isolated compounds as shown in Fig. 1. The isolation of pure withanolides/glucowithanolides was done with the help of IR, NMR and MS data [18,19].

2.4. HPLC analysis of isolated compounds

Pure compounds (1 mg each) were dissolved in HPLC grade MeOH, 10 μL of each sample was used for estimation of purity by HPLC. The Water HPLC system comprising of two waters 515 HPLC pumps, automatic sampling unit (waters 717 plus auto sampler), column oven, photodiode array detector (waters 2996), Merck Rp-18 column (5 μm, 250 × 4.00 mm ID), temperature control module II and Waters Empower software was used for data analysis and data processing.

2.5. Animals

Male Wistar rats, 20–24 weeks old with a weight range of 320–360 g and female balb/c mice, 10–12 weeks old weighing 24–28 g at the start of the experiment were housed in a temperature-controlled colony room under light/dark cycle. These were given access to pellet food and water *ad libitum* throughout the experiment. The behavioural experiments were carried out between 11 a.m. and 4 p.m. This study protocol and the number of animals required for the experiment was approved by Institute's Animal Ethics Committee and the national guidelines on the care and use of laboratory animals were followed.

2.6. Chemicals

Streptozotocin, Dimethylsulfoxide, Aprotinin, Phenylmethylsulfonyl fluoride, NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$ and NaH_2PO_4 (Sigma Aldrich), Tween 20 (Santa Cruz), Fluoroisothiocyanate (FITC)-labeled CD3 anti-rat monoclonal antibody, Phycoerytherin (PE)-labeled CD19 anti-rat monoclonal antibody, Phycoerytherin (PE)-labeled IFN-gamma anti-rat monoclonal antibody, FACS lysing solution, FACS permeabilising solution, (BD Biosciences); Phosphate buffer saline, ELISA kits: TNF-α, IL-1β, IL-6, MCP-1, NO, β- and γ- secretase were used from R & D systems. All other reagents used were of analytical grade.

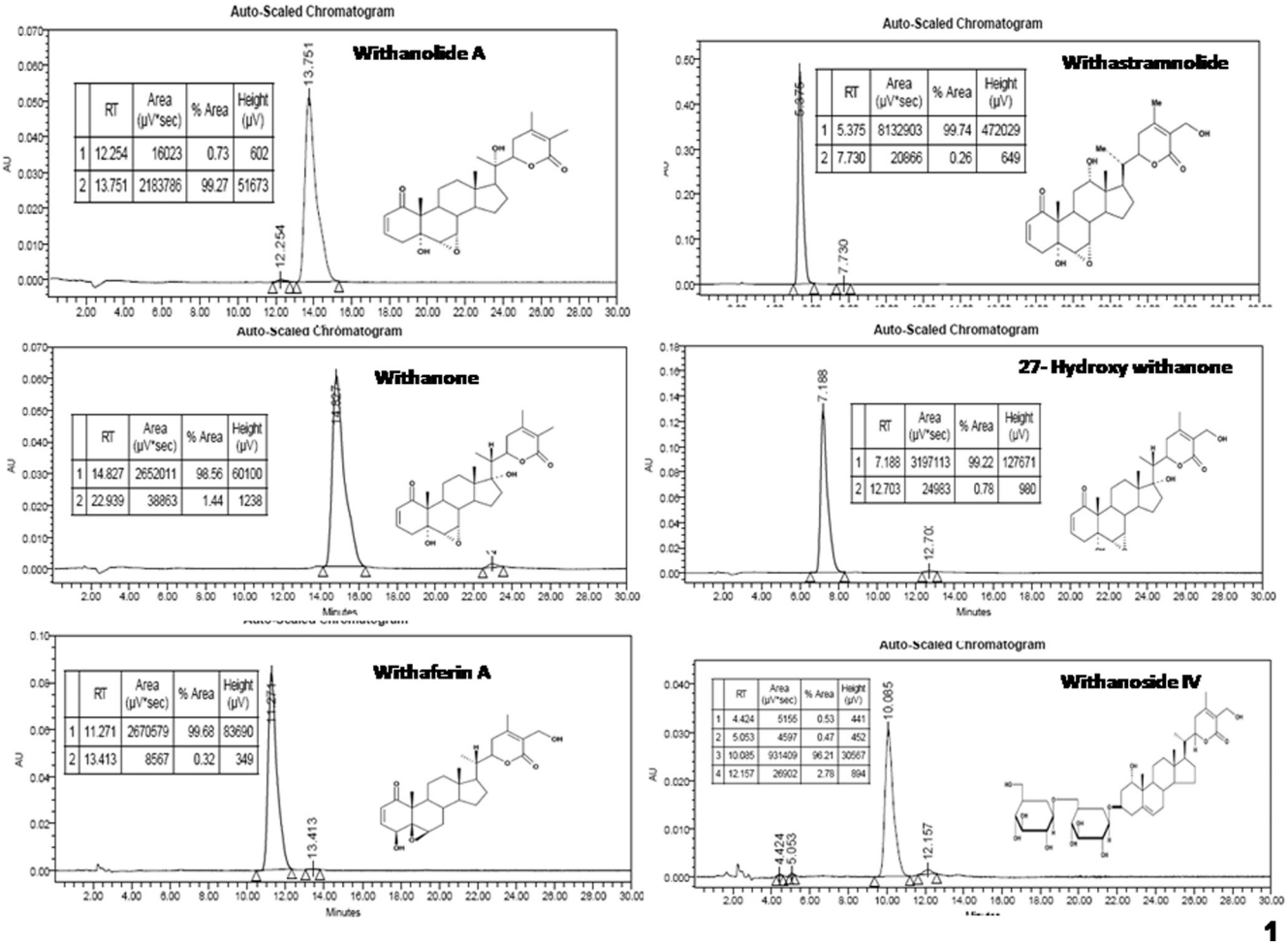


Fig. 1. HPLC chromatograms showing purity profile of Withanolide A (WS-1), Withanone (WS-2), Withaferin A (WS-3), Withastramnolide (WSC), 27- Hydroxy withanone (WSCO) and Withanoside IV (WSG-3).

3. Experimental procedures

3.1. Acute oral safety study

Acute oral toxicity studies were carried out and OECD guidelines no. 423 (OECD) was followed after approval from the Institutional Animal Ethics Committee (IAEC). Three female Balb/C mice, fasted 3–4 h prior to the test, were used for each step and observed individually after dosing during the first 30 min, and periodically during the first 24 h, with special attention for the first 4 h, and daily thereafter, for a total of 14 days. Simultaneously, general behavior and any toxic or untoward symptoms produced by the test material were observed for 14 days for routine pharmacological parameters.

3.2. In-vitro screening of Withanolide a (WS-1), Withanone (WS-2), Withaferin a (WS-3), Withastramnolide (WSC), 27- Hydroxy Withanone (WSCO) and Withanoside IV (WSG-3) in Aβ induced toxicity in PC-12 cell line

The screening of different active constituents was carried out by evaluating the survival of PC-12 cells against Aβ toxicity in presence of the test samples. PC-12, a rat pheochromocytoma, was seeded at a density of 1×10^4 cells/cm^2. The cultures were placed in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. PC-12 cells were incubated and differentiated by the addition of nerve growth factor (NGF) for 48 h. [20] Cell viability was determined by the MTT (3,(4,5- dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide) assay as described previously [21]. The cells were cultured in growth medium for 48 h in 96-well multi plates and then pre-incubated with or without the test samples for 24 h following incubation with Aβ for another 24 h. The cells were stained using 0.05% MTT. After incubation for 4 h at 37 °C the cells were lysed by the addition of lysing buffer [20% SDS, 50% N,N-dimethyl formamide (DMF), pH 4.7] and applied to a microplate reader at 570 nm.

3.3. In-vivo study design

Following six groups of rats were employed in the study with each group comprising of eight animals: 1. Sham-operated group (Sham Control), 2. CSF Control group (CSF Control) that received bilateral ICV injection of artificial CSF (ACSF) (10 µl on each side) as the solvent of STZ 3. STZ-injected group (STZ Control) which received ICV injection of STZ (10 µl on each side) and Groups 4, 5 and 6 were the drug treated groups receiving 5, 10 and 20 mg/kg of WS-2 respectively from day 0 to day 21 after the surgery (Fig. 2). Drugs for oral administration were freshly prepared in 1% w/v acacia gum and administered orally to rats once a day for the duration of the experiment. The above said doses were taken up for the study after carrying out initial experiments on passive avoidance test for memory retention deficit and evaluating its recovery with a multiple range of dose levels of WS-2 (data not shown) and the range of oral doses that showed optimum effect were taken up for the study.

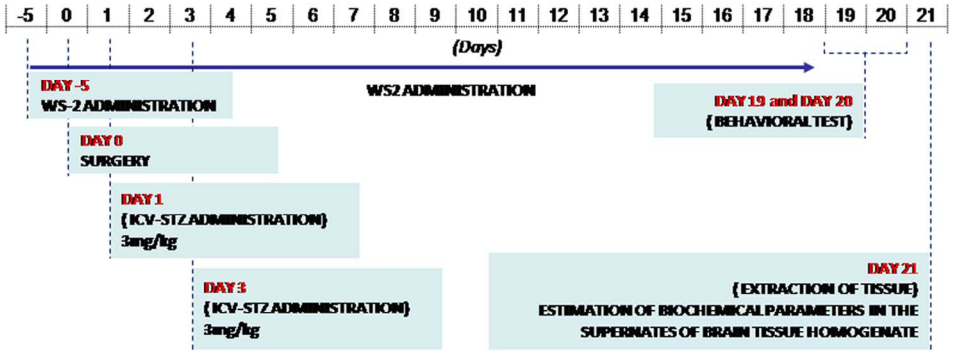


Fig. 2. Schematic representation of in vivo study design to evaluate the anti-Alzheimer activity of WS-2.

3.4. Intracerebroventricular injection of streptozotocin

The anesthetized rats were placed in a Stoelting stereotaxic apparatus (Ugo Basile, Italy) (incisor bar 3.3 mm, ear bars positioned symmetrically). The scalp was cleaned with iodine solution, incised on the midline and a burr hole was drilled through the skull 0.8 mm posterior to bregma, 1.4 mm lateral to saggital suture, and 3.4 mm beneath the surface of brain, according to the stereotaxic atlas [22]. STZ and WS-2 treated STZ groups were administered a bilateral ICV injection of freshly dissolved STZ (3 mg/kg) in cold artificial CSF at a volume of 10 µl on each side. The injection was repeated on day 3. In the CSF Control group, only artificial CSF (120 mM NaCl; 3 mM KCl; 1.15 mM $CaCl_2$; 0.8 mM $MgCl_2$; 27 mM $NaHCO_3$; and 0.33 mM NaH_2PO_4, pH 7.2) was ICV injected. Post-operative, intensive care was taken until spontaneous feeding by the experimental animals was restored.

3.5. Behavioural tests

3.5.1. Single trial passive avoidance test

Deficit in retention of memory was evaluated by a step through passive avoidance apparatus according to the method previously described by Mojard et al. (2007) [23] on days 19th and 20th after 1st injection of STZ. On the acquisition trial, each rat was placed in the lighted chamber and after one minute of habituation period the guillotine door separating the lighted and dark chamber was opened, and initial latency (IL) to enter the dark chamber was recorded. Immediately after the rat entered the dark chamber, the guillotine door was closed and an electric foot shock (75 V, 0.2 mA, 50 Hz) was delivered to the floor grids for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. Twenty-four hours later, the retention latency (RL) time was measured in the same way as in the acquisition trial; the latency time was recorded to a maximum of 600 s.

3.5.2. Elevated plus-maze

The plus-maze consists of two opposite open arms (50 × 10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls. The arms are connected by a central square (10 × 10 cm). On day 7, rats were placed individually at one end of an open arm, facing away from the central square. The time taken for the rat to move from the open arm and enter into one of the closed arms was recorded as 'initial transfer latency' (ITL). [24]. The animal was allowed to explore the maze for 30 s after recording the ITL and returned to its home cage. Then, 4 and 8 days after ITL, the rat was similarly placed on the open arm and the retention latency was noted again and termed as 'first retention transfer latency' (1st RTL) and 'second retention transfer latency' (2nd RTL).

3.6. Biochemical tests

3.6.1. Tissue preparation

On day 21, blood of the experimental animal was taken from the retro-orbital plexus for cell surface marker study and cytokine estimations. The brain of the animals was removed and rinsed with ice-cold isotonic saline. To the tissues was added 4 ml/g tissue of extraction buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 0.05% Tween 20 in phosphate buffered saline. Tissues were homogenized on ice with a polytron and the homogenate was centrifuged at 5000*g* for 15 min. Aliquots of the supernatant were separated and used for biochemical analysis. Supernatants were stored at −80 °C until cytokine analysis [25].

3.6.2. Quantification of Amyloid-beta in supernatant from tissue homogenate

Aβ was measured in brain extracts using a well-characterized sandwich ELISA [26–28]. Aβ was captured using antibody coated plates, followed by detection of Aβ peptides with the horseradish peroxidase-conjugated antibodies. Plates were developed using TMB as substrate (reaction stopped by addition of 6% phosphoric acid) and optical density read at 450 nm. Raw data were converted to pg/mol Aβ by comparison to a standard curve of synthetic Aβ.

3.6.3. Quantification of IL-1β, TNF-alpha, IL-6 and MCP-1 in supernatant from tissue homogenate

On day 21st the samples from different groups of animals were prepared for the analysis of cytokines and mediators as described above. IL-1β, TNF-α, IL-6 and MCP-1 were estimated using commercially available kits based on sandwich and competitive ELISA technique (R & D systems, MN, USA) according to the manufacturers' instructions. All cytokine concentrations were carried out by means of colorimetric measurement at 450 nm on an ELISA plate reader (Multiskan, Thermo Electron Corporation, MA, USA) by interpolation from a standard curve [25].

3.6.4. Acetylcholine (ACh) assay

The neurotransmitter acetylcholine plays a very important role in supporting learning and memory processes in the hippocampus. ACh was estimated in the brain tissue homogenate using the quantitative sandwich enzyme immunoassay technique. Antibody specific for ACH was pre-coated onto a microplates and any ACH present in the sample is bound by the immobilized antibody. The concentration was calculated using standard curve [29].

3.6.5. Estimation of β-secretase and γ-secretase

The tissue samples from all the experimental groups were tested for β- and γ- secretase activity by the addition of a secretase – specific peptide conjugated to the reporter molecules EDANS (5-((2-Aminoethyl)amino) naphthalene-1-sulfonic acid) and DABCYL 4-(4-dimethylaminophenyl) diazenylbenzoic acid. In the uncleaved form the

fluorescent emissions from EDANS is quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing the release of a fluorescent signal. Both β- and γ- secretase were estimated using commercially available kits based on sandwich and competitive ELISA technique (R & D Systems, MN, USA) according to the manufacturers' instructions.

3.6.6. Measurement of oxidative stress parameters

Lipid peroxidation was estimated as the level of malondialdehyde using kits based on sandwich ELISA technique (Cusa Bio) according to the manufacturers' instructions. Nitric oxide and Catalse were also estimated in the brain tissue homogenate of the experimental animals using ELISA Kits.

3.7. Estimation of peripheral intracellular cytokines

The detection of cytokines was performed as per method of Bani et al., 2005; 2006 [30,31]. 80 microliters of peripheral blood was taken and treated with FACS lysing solution. The samples were washed, permeabilized and stained with FITC and PE coupled anti-rat Th1/Th2/Th17 cytokines.

Th1 Cytokines (IFN-γ and IL-2): PE labeled monoclonal antibody IFN-gamma and FITC labeled monoclonal antibody IL-2 were used to quantify the presence of Th1 cytokines.

Th2 Cytokines (IL-4 and IL-10): PE labeled monoclonal antibody IL-4 and FITC labeled antibody IL-10 were used to see the expression of Th2 cytokines. The stained cells were acquired using flow cytometer. Cell quest software was used for gating and calculations. Gating for lymphocytes using forward/sideward scatter was facilitated by staining. The resulting numbers were percentages of cytokine expression of those subpopulations.

Th17 Cytokines (IL-17 and TGF-β): PE labeled monoclonal antibody IL-17 and FITC labeled monoclonal antibody TGF-β were used to see the expression of Th17 cytokines in the peripheral blood of experimental animals.

3.8. Statistical analysis

The mean and standard error (S.E.) of the mean for each experimental group was calculated and results expressed as percent inhibition compared with the control group. The significance of the study was determined statistically by applying one-way ANOVA.

4. Results

4.1. Acute oral safety study

Neither mortality nor any untoward symptoms was observed in animals treated with WS-2 up to 1000 mg/kg p.o. There was no change in general behavior when compared to the animals of normal control group.

4.2. In-vitro screening in PC-12 cell line

MTT Assay was used to determine the viability of PC12 cells for visualizing the effect of different active constituents on Aβ- induced cytotoxicity. Fig. 3 shows the percentage survival of the cells in presence of WS-1, WS-2, WS-3, WSC, WSCO and WSG-3. The maximum survival of the cells was observed in the plate treated by WS-2 which was 35.17% when compared with 7.82% of the control. The data shows that WS-2 shows significant protective effect against Aβ toxicity.

4.3. Behavioural tests

4.3.1. Single trial passive avoidance test

As WS-2 was the most significant active constituent in the *in vitro* screening the in vivo experiments for anti-Alzheimer's activity evaluation were carried out at graded oral doses of WS-2. The mean initial latency on day 19th did not differ significantly between the sham, vehicle-treated, ICV STZ group and WS-2 at 5, 10 and 20 mg/kg treated ICV STZ group. On day 20th the mean retention latency in ICV STZ group was significantly less 103 ± 24.9 s as compared to that of sham rats (302.73 ± 22.1 s). The group that was treated with WS-2 at doses of 5, 10 and 20 mg/kg p.o, showed significant reversal of transfer latency. In WS-2 20 mg/kg the mean retention latency was 381.09 ± 32.3 s, which was significant indicating improved retention of memory (Fig. 4).

4.3.2. Elevated plus-maze

Significant decrease in transfer latency in all groups (including control) on the second day was obtained. The analysis revealed significant differences in transfer latency in elevated plus maze performance between WS-2 (20 mg/kg) and ICV-STZ treated animals on both the days (Fig.5).

4.4. Biochemical tests

4.4.1. Quantification of Amyloid-beta in supernatant from brain tissue homogenate

The brain tissue homogenate was subjected to quantification by commercially used Amyloid beta ELISA kit. Administration of WS-2 at graded doses decreased the Aβ concentration from 228.76 ± 12.4 pg/ml observed in ICV-STZ control group to 126.38 ± 8.1 in WS-2 at 20 mg/kg treated dose (Fig. 6).

4.4.2. Quantification of IL-1β, TNF-alpha, IL-6 and MCP-1 in supernatant from tissue homogenate

WS-2 at graded doses of 5, 10 and 20 mg/kg significantly decreased the TNF-α, IL-1β, IL-6 and MCP-1 (Fig. 7) levels in a dose dependent manner showing significant inhibition TNF-α, IL-1β, IL-6 and MCP-1 parameters at higher dose levels of 10 and 20 mg/ kg per oral.

4.4.3. Acetylcholine assay

There was a significant decrease in the brain Acetylcholine levels in ICV-STZ animals compared with the ACSF-injected and Sham operated rats. However, oral administration of WS-2 (5, 10 and 20 mg/kg) reversed the reduction in acetylcholine activity compared with the STZ -injected group. The higher doses of WS-2 (10 and 20 mg/kg, p.o.) treatment showed marked effect in restoring the acetylcholine activity compared with the lower dose. (Fig. 8).

4.4.4. Estimation of β-secretase and γ-secretase

Oral treatment of WS-2 at graded doses of 5, 10 and 20 mg/kg resulted in the decreased concentration of both β- and γ- secretase. The fluorometric reaction was proportional to the level of secretase enzymatic activity in the tissue preparation of the experimental groups (Fig. 9a and b).

4.4.5. Effect of WS-2 on oxidative stress parameters

Neural cells attacked by free radicals because of oxidative stress has calamitous role in neurodegeneration. Lipid peroxidation expressed through the concentration of malondialdehyde, nitric oxide and catalase were evaluated as oxidative stress parameters in the brain tissue homogenate of the experimental animals. The elevated levels of the oxidative stress parameters due to ICV administration of STZ in STZ control group was dose dependently inhibited by the oral treatment of WS-2 at the dose levels of 5, 10 and 20 mg/kg, showing the most significant effect at the highest dose of 20 mg/kg (Table 1).

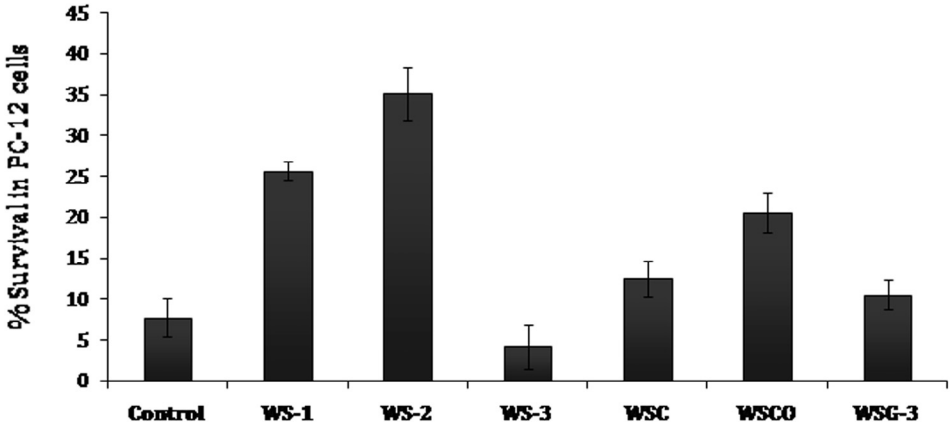


Fig. 3. Quantitative analysis of cell viability regulation by Withanolide A (WS-1), Withanone (WS-2), Withaferin A (WS-3), Withastramnolide (WSC), 27-Hydroxy withanone (WSCO) and Withanoside IV (WSG-3) following exposure to Aβ.

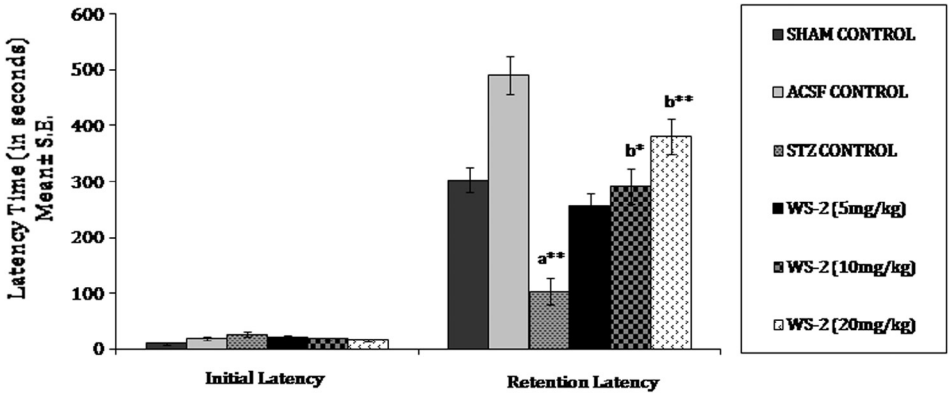
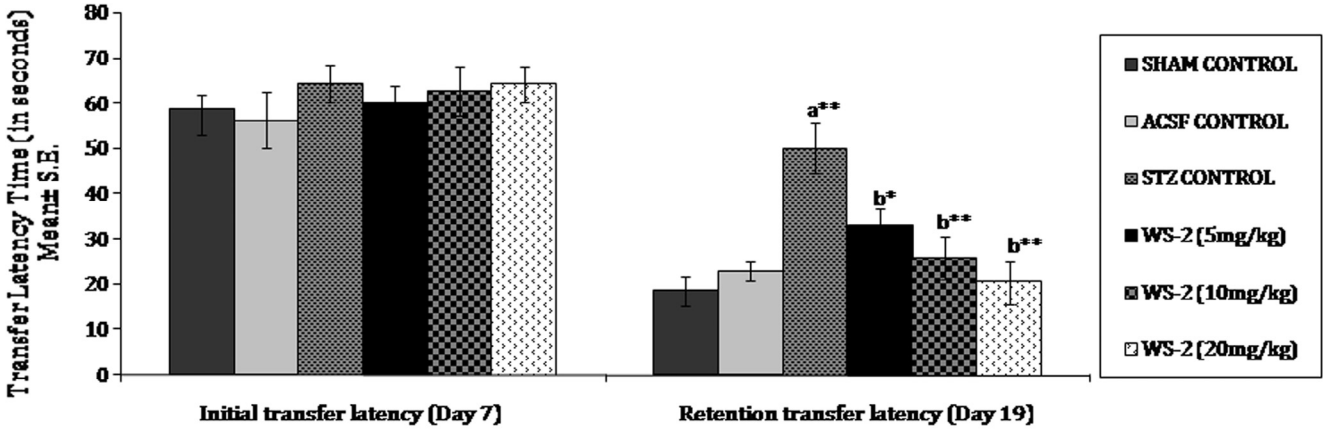


Fig. 4. The effect of WS-2 treatment (5, 10 and 20 mg/kg/ p.o.) on passive avoidance performance after ICV injection of STZ in rats as indicated by initial and retention latencies.

p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

4.5. Estimation of peripheral intracellular cytokines

Significantly higher peripheral blood cytokine concentrations were detected in ICV-STZ group when compared to sham control. Fig. 10 shows the elevated expression of Th1 cytokines IFN-gamma and IL-2 and Th17 cytokines IL-17 and TGF-β; and depletion of anti-inflammatory cytokines IL-4 and IL-10 was observed in STZ control. The peripheral cytokine level in graded doses of WS-2 treated animals showed modulation of the cytokine expression. The Th1 and Th17 cytokine expression was decreased in WS-2 treated group when compared to STZ-control, however, there was non-significant increase in the expression of Th2 cytokine expression. The elevated levels of peripheral proinflammatory cytokines have important biological implications in AD in view of our recent understanding of peripheral immune system to brain communication affecting mood, cognition and behavior.



p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

Fig. 5. Effect of WS-2 (5, 10 and 20 mg/kg/ p.o.) on spatial memory in elevated plus maze test in rats.

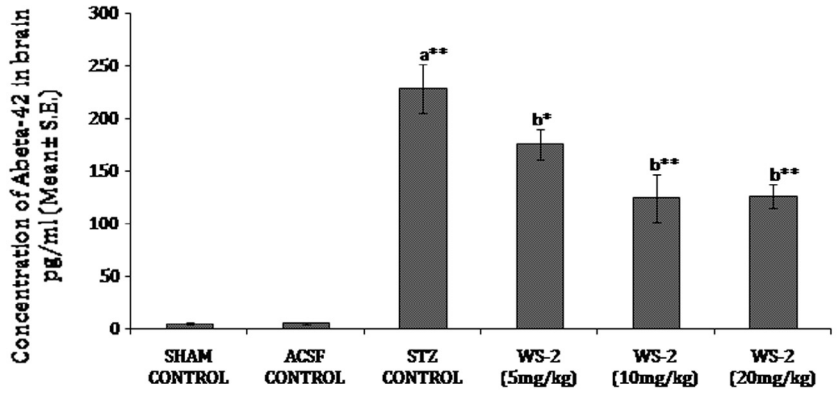


Fig. 6. The graph represents the dose dependent effect of WS-2 on expression of Aβ-42 in supernatant from brain tissue homogenate in ICV STZ treated rats.

p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

5. Discussion & conclusion

With the increase in life expectancy due to health care there is sharp increase in the older patient base raising specific concerns about the increase in the age related degenerative diseases specifically associated with the brain [32–34]. AD is one of the biggest future threats to human health and well being. As many pathogenetic factors are implicated in the disease, the current hitting-one-target therapeutic strategy is proved inefficient to AD. As a result, finding multi-potent agent that aims at multiple targets is attracting more and more attention.

Researchers at the National Brain Research Centre (NBRC), have conducted studies on mice that suggest Ashwaganda extract may reverse memory loss and improve cognitive abilities in those with the disease [35] .Our study showed Withanone (WS-2) to be the most active constituent responsible for a multi-dimensional benefits against AD. Such considerations have fundamental implications for the design of targeted pharmacological therapy in AD. WS-2 significantly prevents the cognitive impairment and attenuates the inflammation and oxidative stress in ICV STZ model in rats (Fig. 11). WS-2 was tested at doses of 5, 10 and 20 mg/kg, the most effective dose being 20 mg/kg after which there was no further enhancement in the activities and plateau state was obtained.

Amyloid composed primarily of Aβ from the neuritic plaques in the Alzheimers affected brain of patients. Aβ is approximately 4-kDa peptide derived from the amyloid precursor protein. WS-2 inhibited the production of Aβ in the brain tissue of the experimental animals (Fig. 6). Reduction in Aβ improved the cognitive function and reduced degradation of memory indicating to have preventive effect in AD. WS-2 at the higher dose level of 10 and 20 mg/kg p.o. improved the latency time/retention time in the passive avoidance and elevated plus maze test in STZ induced memory deficit in animals (Figs. 4 and 5). ICV administration in rats has been linked to sporadic AD in humans and the cognitive impairment is associated with free radical generation in this model [36]. Better understanding of the mode of action of WS-2 implicated in AD improves research in designing therapy specifically targeted against the inhibition of immune mediated inflammatory processes.

Present day science provides us with evidences supporting the involvement of inflammation as one important cascade in developing and worsening of AD. Inflammation in brain is promoted by deposition of β-amyloid resulting in neuronal damage and death [37,38]. Daily oral dose of WS-2 reduced the increased levels of pro-inflammatory mediators TNF-α, IL-1β, IL-6 and MCP-1(Fig. 7).

The neurotoxicity exerted by aggregated Aβ can be mediated by several mechanism, such as the generation of reactive oxygen species, preseneline 1, preseniline2 and activation of signaling pathways. Aβ induces lipoperoxidation of membranes and lipid peroxidation products [39]. Lipids are modified by ROS and there is a strong correlation between lipid peroxides, antioxidant enzymes, and amyloid plaques in AD brains [40]. The elevated levels of the oxidative stress parameters due to ICV administration of STZ in STZ control group was dose dependently inhibited by the oral treatment of WS-2 at the dose levels of 5, 10

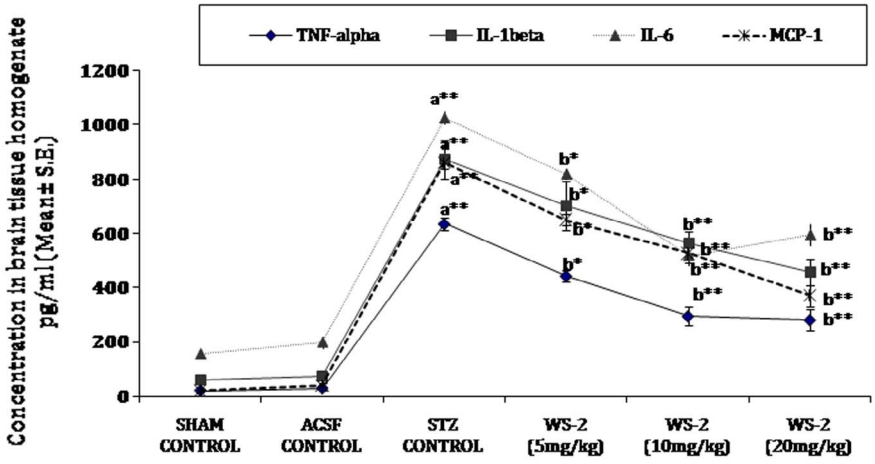


Fig. 7. The graph represents the effect of WS-2 on expression of TNF-α, IL-1β, IL-6 and MCP-1 in supernatant from brain tissue homogenate in ICV STZ treated rats.

p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

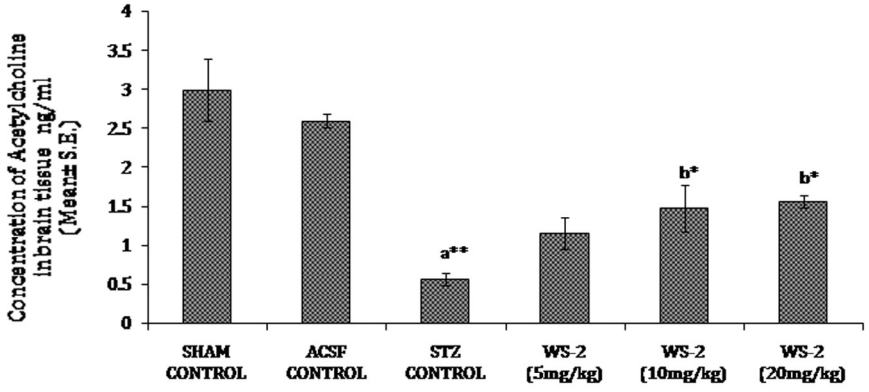


Fig. 8. Data shows the effect of graded doses of WS-2 on expression of Acetylcholine in the brain tissue homogenate estimated through ELISA.

p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

and 20 mg/kg, showing the most significant effect at the highest dose of 20 mg/kg (Table 1).

Experimental evidences exist that show the involvement of cholinergic and glutamatergic in the etiology of AD. Acetylcholine is decreased in both concentration and function, thereby, decreasing the processes of memory and learning. WS-2 restored the depleted levels of acetylcholine against the ICV-STZ control group evaluated in the brain tissue homogenate (Fig. 8).

The production of Aβ requires two sequential cleavages induced by beta- and gamma-secretases on the beta-amyloid precursor protein (APP). Altered activity of these secretases is involved in the pathogenesis of AD. Treatment of experimental animals with WS-2 in graded doses for 21 days caused decrease in enzymatic activity of both β- and γ- sectretase activity, thereby showing significant protective potential (Fig. 9a and b).

Cerebrospinal fluid (CSF) closely reflects the composition of the brain extracellular space, however, CSF is not routinely collected for the evaluation of AD, and lumbar puncture is not a widespread procedure in primary care, psychiatric practice, or geriatric facilities that care for AD patients. Therefore, blood biomarker molecules would be more widely applicable and would reduce the need for invasive, painful, expensive, and time-consuming testing. The degeneration of the brain in AD is due to direct effect produced by cytokines such as IFN-gamma and IL-2 [15] another important TH17 or Treg cytokine-TGF-β is also significantly related to the pathogenesis of Alzheimer's disease. It can either promote or suppress the disease by initiating TH17 or Treg pathway. In addition, overactive microglias secrete TH17 cytokines that include IL-1, TNFα, and IL-6 in AD, especially when they are stimulated

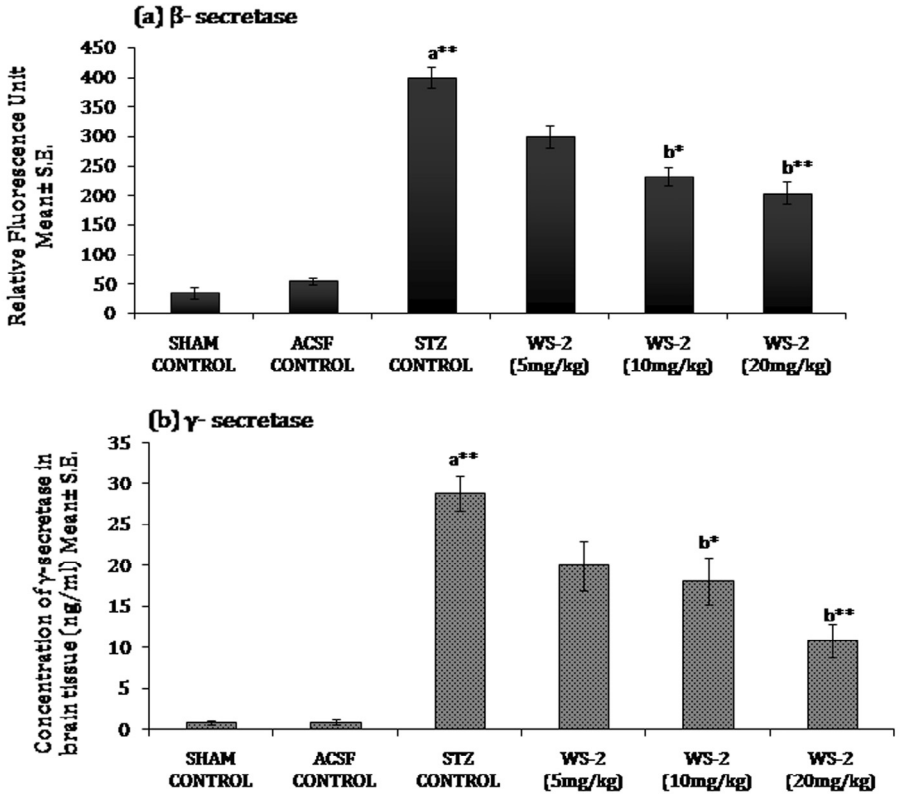


Fig. 9. The graph represents the dose dependent effect of WS-2 on expression of (a) β-secretase and (b) γ-secretase in supernatant from brain tissue homogenate in ICV STZ treated rats.

p-values: *p≤ 0.05, **p≤ 0.01, Asterisks with p value 'a' indicate significant difference of Sham Control vs STZ Control and 'b' indicates WS-2 treated group vs STZ Control; n= 8 per group.

Table 1
Effect of oral treatment of WS-2 on streptozotocin-induced biochemical changes leading to oxidative stress in rat brain.

Treatment	Levels of oxidative stress parameters in brain tissue homogenate (Mean ± S.E.)		
	Lipid peroxidation Malondialdehyde (MDA) pmol/ml	Nitric oxide pg/ml	Catalase mlU/ml
Sham control	54.75 ± 8.31	98.01 ± 5.4	202.94 ± 8.79
ACSF control	71.89 ± 8.8	164.26 ± 9.28	226.66 ± 11.30
STZ control	975.34 ± 19.48[a,**]	3582.92 ± 58.29[a,**]	1568.47 ± 34.55[a,**]
WS-2 5 mg/kg p.o.	759.11 ± 15.04[b,*]	2721.77 ± 23.47[b,*]	1082.55 ± 22.97[b,*]
WS-2 10 mg/kg p.o.	602.85 ± 12.57[b,*]	1563.83 ± 34.51[b,*]	806.62 ± 17.48[b,*]
WS-2 20 mg/kg p.o.	493.17 ± 17.14[b,**]	1154.41 ± 18.86[b,**]	723.09 ± 19.47[b,**]

Asterisks with p value 'a' indicate significant difference of Sham control vs STZ control and 'b' indicates WS-2 treated group vs STZ control; n = 8 per group.
* p-values: p ≤ .05.
** p-values: p ≤ .01.

by amyloid beta. Role of WS-2 was evaluated in modulating the cytokine balance in the blood of the experimental animals. WS-2 inhibited Th1 and Th17 cytokine expression and non-significantly increased the levels of Th2 cytokines (Fig. 10). The CNS disregulation (high levels of proinflammatory cytokines, low levels or activity of anti-inflammatory cytokines) can lead to cytokine production and can induce an amplification cycle of cellular activation and cytotoxicity [41].

Considering the fact that current knowledge about natural products effective in cognitive dysfunction is limited, the presently identified natural multifunctional agents are only the tip of the iceberg. With the increase of information on natural moieties obtained from medicinal plants and the effort of evaluating compound WS-2 from a revered plant *Withania somnifera* paves way for its future exploration as a multifactorial therapeutic agent for AD (Fig. 11). The ensemble of experimental results discussed paves the way towards designing of targeted and selective immunotherapeutic agent for the pharmacological interventions in AD.

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Conflict of interest disclosure

The authors declare no conflict of interest.

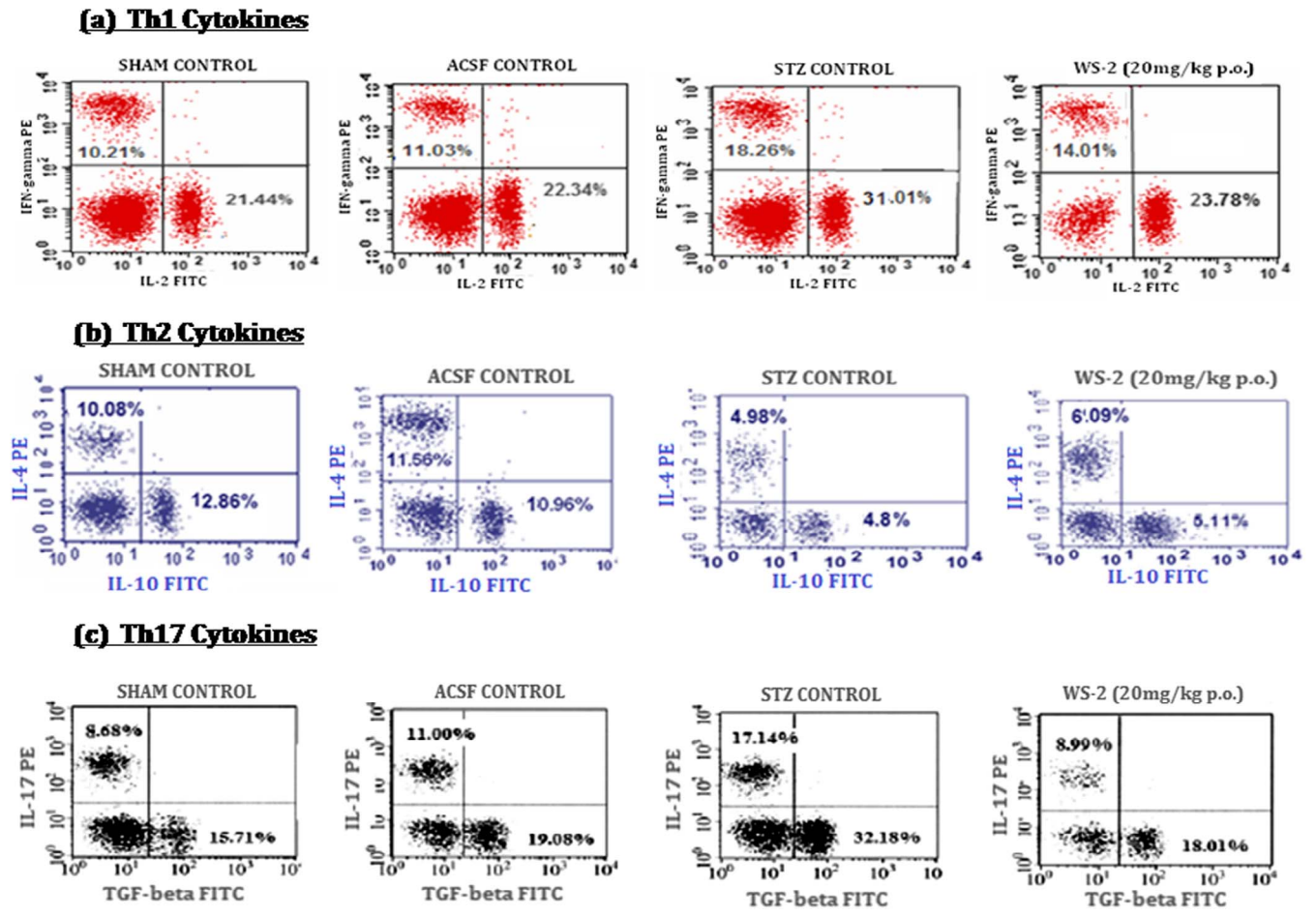

Fig. 10. Flowcytometric quadrant plot representation showing the effect of WS-2 on expression of Th1 Cytokines: IFN-γ and IL-2; Th2 cytokines: IL-4 and IL-10 and Th17 cytokines: IL-17 and TGF-β. The quadrant plot represents the counts for one representative rat from each group and is acquired according to the standard procedures of the BD-LSR flowcytometer.

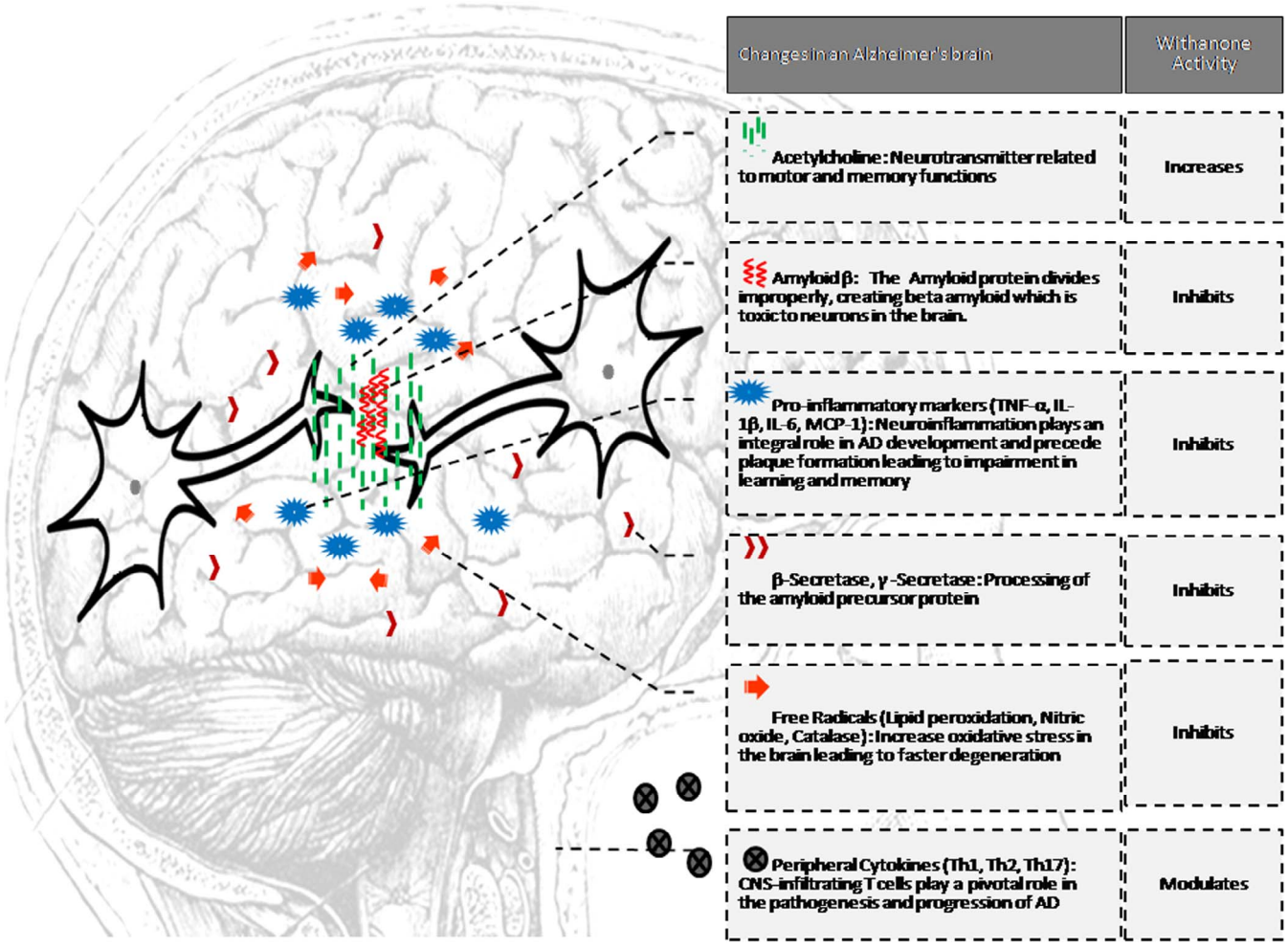

Fig. 11. Graphical representation illustrating mechanism of action of Withanone showing the neuroprotective effect in alleviating memory loss and cognitive dysfunction.

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