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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.