

The Neuroprotective Effect of *Lycopodium* and *Withania Somnifera* on Alzheimer's Disease Induced by Aluminum Chloride in Rats

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Abstract

Alzheimer's Disease (AD) is a serious health problem worldwide and known as the most common type of neurodegenerative diseases. *Lycopodium* (Lyc) and *Withania Somnifera* (WS) contains a bio active compound that proved to restore and improve cholinergic system. The present study aimed to examine the neuroprotective potential of Lyc and WS on Aluminum Chloride induced AD in rats. Fifty female Albino rats were equally and randomly divided to five groups, ten rats in each group. The rats of the first group received distilled water and served as control. The rats from second to fifth group received $AlCl_3$ (175mg/kg), the second groups consider as AD group, in addition to $AlCl_3$, third group received Lyc (50mg/kg), fourth received WS (200mg/kg), fifth received both treatments. After 30 days, the Acetylcholine (Ach), Acetylcholinesterase (AchE), Butyrylcholinesterase (BchE), Amyloid beta-42 ($A\beta$ -42), Tyrosine hydroxylase (TH), and Na^+/K^+ ATPase were evaluated. The result showed that $AlCl_3$ significantly increased Ach, AchE, BchE, $A\beta$ -42 levels, and significantly decreased TH and Na^+/K^+ ATPase levels in rats model of AD. Administration of Lyc and WS to rats model of AD for 30 days showed an improvement in biochemical alteration caused by $AlCl_3$. This study suggests that oral administration of Lyc and WS have a neuroprotective effect in AD animal model.

Keywords: Alzheimer's Disease, *Lycopodium*, *Withania Somnifera*, Cholinergic, Amyloid beta.

Introduction

The neurodegenerative diseases are a progression disorders that occur in the central nervous system (CNS), in 2040 it is expected to excel cancer mortality as reported by the world health organization, and there is 10 million new cases of dementia are diagnosed each year (WHO 2020). Alzheimer's disease (AD) is the most common neurodegenerative disease affect one in nine people whom over 65 years old (Aljarari and Bawazir, 2019). In Saudi Arabia 51.9% of dementia cases are AD patients (Alhazzani *et al.* 2020). Misfolded protein deposition is the leading cause of a variety of neurodegenerative disorders (Rahman *et al.* 2021). The key symptom that can be observed in AD patient is dementia that appears in cognitive dysfunction, memory deficit, learning and language difficulty. The pathogenesis can be characterized by extracellular Amyloid- β ($A\beta$) plaques, intracellular neurofibrillary tangles, neural loss and cerebral atrophy (Wang *et al.* 2015).

One of the pathological markers in the brain of AD is the presence of $A\beta$ in amyloid plaques, $A\beta$ is raised astrocyte activation that leads to synapse destruction and memory degradation is linked to the production of proinflammatory cytokines and reactive oxygen species (ROS) leading to protein and DNA damage (Qusti 2017). Acetylcholine (Ach) is a neurotransmitter that is essential for the proper operation of the central cholinergic system, the enzyme acetylcholinesterase (AchE),

which is responsible for the breakdown of acetylcholine, is found in higher amounts in the brains of AD patients (Uddin *et al.* 2016), Ach deficiency in the brain of AD patients appears to be a major factor in the development of dementia (Kandeda *et al.* 2021). Cholinergic system changes is associated with AD, butyrylcholinesterase (BchE) has been linked to A β plaques and neurofibrillary tangles as seen in human brain tissue (Darvesh, Reid, and Martin 2010). Aluminum (Al⁺³) is a potential metal involve in the pathogenesis of AD because the brain is a main target for it, Al⁺³ can increase the formation of free radical which induce inflammation leading to tissue damage (Vishala, Pitchaiah, and Pravadha 2019). Al⁺³ has high affinity to transferrin so it can across the blood brain barrier (BBB) and accumulate in hippocampus and cortex regions (Zhao *et al.* 2020). Studies were reported that Al⁺³ accumulation in the brain lead to increase in A β accumulation, neuro inflammation and necrosis, the neuro inflammation is known as major risk in neural diseases because it stimulate the microglia which result in hippocampus dependent memory, learning and memory deficiency (Oshima *et al.* 2013; Wang *et al.* 2014; Sadek, Lebda, and Abouzed 2019).

Utilizing traditional herbs that have been used in ancient aged to improve health care got a lot of interest lately. Club moss (*Lycopodium*) belongs to *Lycopodiaceae* family of plants. Lyc has been a popular cure in Chinese and homeopathic medicine. Many studies have been reported that Lyc has an anti cancer, analgesic, and anti-inflammatory activity (Hu, Chandler, and Hanson 1987; Brustolin *et al.* 2017; Paramita *et al.* 2018). In a previous study, Lyc has showed provide neuroprotection against Parkinson's Disease (PD) by reducing ROS production, α -synuclein expression, MMP-mediated glial activation, and consequent neurodegeneration. Since Lyc has an effect on PD it may has a promising protective effect on AD (Jayaraj *et al.* 2019).

Ashwagandha (*Withaniasomnifera*) belong to *Solanaceae* family of plant. It has been used in India for ancient years. Many effective components have been identified in WS known as withanolides including steroid, alkaloids and lactones (Paha *et al.* 2021). Pharmacological researchers reported that WS has anti-inflammatory, antioxidant, anti anxiety, and improve cardiovascular activity (Kulkarni and Dhir 2008; Bano *et al.* 2015). It was reported that WS contain Withanolides, a group of steroidal lactones, and Withanamide A and C which are mostly responsible in protective effect against free radicals damage, Withanamide binds to A β , inhibit fibril formation in the neurons and reduce A β toxicity to the cells (Ahmed *et al.* 2013; Ovaisy *et al.* 2018). Many previous studies were demonstrated that WS successfully ameliorated neurodegeneration, cognitive decline, synaptic plasticity impairments in rats and mice model of cognitive decline and neurotoxicity (Elhadidy *et al.* 2018; Gupta and Kaur 2019; Ghosh *et al.* 2019).

Relatively few studies have examined the effect of Lyc on AD and none has discovered the effect of the mixture of Lyc and WS on AD, Therefore, this study was aimed to investigate the beneficial effects of oral administration of Lyc and WS, and the mixture of both on the A β -42, neurotransmitter enzymes: Ach, AchE, BchE, Tyrosine hydroxylase, and Na⁺/K⁺ATPase in AlCl₃ induced AD in rats.

Materials and Methods

Animals

50 female albino rats weighing 150–200 g were procured from the experimental animal house of King Fahad Medical Research Center (KFMRC), King Abdulaziz University (KAU), Jeddah, Kingdom of Saudi Arabia (KSA). The prior permission for this experiment was obtained by the biomedical research ethics committee of the Faculty of Medicine, King Abdulaziz University (KAU), Jeddah, (KSA) with the registered NO. (545-20). Animals were housed in cages and re-

ceived normal rat chow and tap water *ad libitum* in a constant environmental (room temperature 25 °C, room humidity 68) with a 12 h light and 12 h dark cycle.

Chemicals

Aluminum Chloride was purchased from Sigma Aldrich, Sant Louis, United State America (USA), Supplied as a white powder and diluted in distilled water for oral administration.

Plants Material

Lycopodium clavatum (Lyc) was purchased from Health Embassy LTD, Cheltenham, United Kingdom. *Withania Somnifera* (WS) was purchased from R V Essential, NarainaVihar, India.

Plants extracts preparation

2.5 g of Lyc leaves and 10 g of WS roots were crushed separately via grinder machine to get fine powder then the extracts were prepared by suspending each dry plants powder in 100 ml of distilled water and the suspension was stirred at 45°C overnight in shaker incubator. The obtained extract was filtered first over cheesecloth then over Whatman® filter paper and the filtrate was collected, then water was removed using a rotary evaporator (HS-2005S) at 6 °C to obtain a semi-dry extract. Then these extract transferred into the freeze dryer vacuum overnight to obtain the full-dry extract. It is then kept at 4 °C and used when starting the experiment with dissolving it in an appropriate amount of distilled water according to the used dose (Manchanda *et al.* 2017).

Experiment design

Rats were divided into five groups, ten rats in each group as below:

Table 1. Study Design

Groups	Control	AD	Lyc	WS	AD Lyc-WS
Distilled water	√				
AlCl ₃ 175mg/kg (Liu <i>et al</i> 2020).		√	√	√	√
Lyc 50mg/kg (Jayarajet <i>al.</i> 2019).			√		√
WS 200mg/kg (Shivamurthy, Manchukonda, and Ramadas 2019).				√	√

After a month, all rats were anesthetized through Isoflurane and scarified through cervical dislocation, brains were removed and washed with PBS (0.01 M, pH 7.4) at 2-8°C . Absorbed the water with filter paper and weigh, homogenized, then centrifuge the tissue homogenate for 10 min at 10000 g at 4°C and immediately frozen in -80 °C for assays.

Biochemical Studies

All the assay kits and ELISA kits were used for the measurement of AchE (Cat. #E-EL-R0355), Ach (Cat. #E-EL-0081), BCHE (Cat. #SEC348Ra), TH (Cat. #E-ELR1437), Na⁺/K⁺ AT-Pase (Cat. #E-BC-K539-M), and Aβ1-40 (E-EL-R3030) were purchased from Elab Sciences Biotechnology, USA. All kits procedures were performed according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis is done by using R software (4.0.4). Shapiro-Wilk test of normality was used to test if each group under each parameter follows the normal distribution or not. One way ANOVA was used to show if there is significance among groups. Kruskal-Wallis (K) test is used to test if there is a significance difference among groups followed by Mann-Whitney test to compare the difference among two groups. Finally, variables described by mean±standard_deviation, p-Value less than 0.05 considered significant.

Results

Effect of Lycopodium and Withania Somnifera on Neurotransmitter enzymes activity in AlCl₃ induced AD in rats

It was observed that Ach, AchE and BchE were significantly ($P < 0.05$) decreased in AD group compared to control group. As seen in Table 2, these enzymes activity were decreased significantly ($P < 0.05$) in the treatments of Lyc, WS and their mixture groups compared to AD group.

Table 2. The Effect of Lyc and/or WS on cholinergic enzymes in AD induced by AlCl₃ in rats

Groups	C	AD	Lyc	WS	AD Lyc-WS
Ach(pg/ml)	20.7±0.82	65.1±0.74	45.1±0.74 ^{***###^^^}	38.3±0.71 ^{***###^^^}	28.1±0.74 ^{***###}
AchE(ng/ml)	9.82±0.22	51±0.82 ^{***}	16.9±0.74 ^{***###^^^}	13.6±1.01 ^{***###}	13.4±0.84 ^{***###}
BchE(ng/ml)	30.5±1.38	72.4±1.71 ^{***}	48±0.67 ^{***###^^^}	41.1±0.60 ^{***###^^^}	34.8±0.63 ^{***###}

Values were expressed as mean±std. There is a significant difference from control values at $P < 0.05^*$, 0.01^{**} , 0.001^{***} , There is a significant difference from AD values at $P < 0.05^\#$, $0.01^{##}$, $0.001^{###}$ and There is a significant difference from AD Lyc-WS values at $P < 0.05^\wedge$, $0.01^{^^}$, $0.001^{^^^}$.

Effect of Lycopodium and Withania Somnifera on Amyloid beta-42 in AlCl₃ induced AD in rats

Table 3 represents the effect of Lyc and/or WS on A β -42 level in AlCl₃ induced AD groups. As statistically assessed, The level of A β -42 in AD group has significantly ($P < 0.05$) raised when compared to the control group. AD groups treated with Lyc, WS or their mixture showed significantly ($P < 0.05$) decreased levels in compared to AD untreated group. The group that treated with both plants showed a lower level among treatments group and close to control.

Table 3. The effect of Lyc and/or WS on Amyloid beta-42 in AD induced by AlCl₃ rats

Groups	C	AD	Lyc	WS	AD Lyc-WS
A β -42 (pg/ml)	45.3±3.44	77.2±6.81 ^{***}	65.5±0.85 ^{***###^^^}	56.7±1.39 ^{***###^^^}	41±0.82 ^{***###}

Values were expressed as mean±std. There is a significant difference from control values at $P < 0.05^*$, 0.01^{**} , 0.001^{***} , There is a significant difference from AD values at $P < 0.05^\#$, $0.01^{##}$, $0.001^{###}$ and There is a significant difference from AD Lyc-WS values at $P < 0.05^\wedge$, $0.01^{^^}$, $0.001^{^^^}$.

Effect of Lycopodium and Withania Somnifera on Tyrosine Hydroxylase in AlCl₃ induced AD in rats

To explore the effect of Lyc, WS and if their mixture can protect dopaminergic neurons from damage in AD, TH activity was measured in the brain of AD rats. As shown in Table 4, TH level significantly decreased in AD group in compared to the control group. Treatment of AD groups with Lyc, WS and their mixture showed a significant increase in TH levels in compared to AD untreated group. In addition, the data demonstrated in Table 4 showed a significant increase in Na⁺/K⁺ATPase activity in the brain of AD group when compared to control group. AD group that treated with Lyc and WS showed a lower level of Na⁺/K⁺ATPase compared to AD group. Levels of TH and

Na⁺/K⁺ATPase in both AD Lyc-WS groups showed a better results than treated separately with Lyc or WS.

Table 4. The effect of Lyc and/or WS on TH and Na⁺/K⁺ATPase in AD induced by AlCl₃ rats

Groups	C	AD	Lyc	WS	AD Lyc-WS
TH (ng/ml)	5.8±0.36	1.46±0.05 ^{***}	4.23±0.09 ^{***###}	3.42±0.45 ^{***#####^}	4.26±0.51 ^{###}
Na ⁺ / K ⁺ ATPase (umol/ml/hr)	0.21±0.01	0.54±0.03 ^{***}	0.41±0.01 ^{***#####^}	0.39±0.01 ^{***#####^}	0.27±0.02 ^{***###}

Values were expressed as mean±std. There is a significant difference from control values at P<0.05^{*}, 0.01^{**}, 0.001^{***}, There is a significant difference from AD values at P<0.05[#], 0.01^{##}, 0.001^{###} and There is a significant difference from AD Lyc-WS values at P<0.05[^], 0.01^{^^}, 0.001^{^^^}.

Discussion

AchE degrade Ach to yield choline and acetate acid, BchE plays a role in cholinergic neurotransmission with AchE (Jasiecki, Targońska, and Wasąg 2021), Ach hydrolysis increased by increasing AchE activity and affect cholinergic neurotransmission process which linked to AD (Čolović *et al.* 2013). Al³⁺ has completely linked to AD, Al³⁺ can accumulate in the brain and affect permeability and integrity of the BBB in many ways on of them by disturbing the cholinergic enzymes (Favarato *et al.* 1992; Aljarari and Bawazir 2019). The result of the present study showed that AlCl₃ increased Ach, AchE and BchE in the brain of AD rats model in comparison to control group. This increase may due to the neurotoxic effect of AlCl₃ to the brain which increase AchE hydrolysis to Ach and increase Ach level as a result as seen in the progression of AD in rats administrated AlCl₃. This result coincided with the result of previous studies (Kaizer *et al.* 2008; Khalifa *et al.* 2020; Obafemi *et al.* 2021).

Treatment of AD with Lyc or WS separately or in mixture could moderate AlCl₃ induced cholinergic deficits by reducing acetylcholine degradation, as seen in decreased levels of Ach, BchE activity and restored AchE level in the treated groups in comparison to AD group. This result may due to that Lyc contains Huperzine A which is a bioactive alkaloid compound that inhibit AchE and BchE activity and its pharmacodynamic properties similar to available AD drugs (Szypuła and Pietrosiuk 2021). As suggested by Konrath *et al.* (2012), this alkaloids in Lyc extracts can cross the BBB and bind to the enzyme–substrate complex and enzyme active center, inhibiting AchE activity will result in a delay in Ach breakdown and reduce its level. As regards the role of WS in the treatment of AD, the present study has demonstrated that WS has been shown to have a neuroprotective effect that could be used as a therapeutic agent for the treatment of AD by decreasing the activity of Ach and AchE, this result is in accordance with previous research has shown that WS improves cholinergic activity and protects against scopolamine-induced memory loss (Gautam, Wadhwa, and Thakur 2016). From this data it can revealed that both plants have a potential effect on the cholinergic enzyme and prevent cholinotoxicity of AlCl₃ in AD rats model.

Brain injury play a pivotal role in the Aβ deposition of AD pathology (Zhao *et al.* 2020). High Aβ-42 content was correlated with dementia, when Aβ-42 increased, fibrillar brain amyloid increased which is a high risk and correlated with AD (Lopez *et al.* 2019). Aβ stimulates the development and release of inflammatory mediators and NO, which can activate microglia's and astro-

cytes, and trigger inflammatory reactions. Also, it may cause nerve cell death and impair the automatic regulation of vasomotion, both of which would exacerbate ischemic cerebral injury (Zhao, Zhao, and Zhang 2015). The results of this study revealed that oral administration of $AlCl_3$ can elevate $A\beta$ -42 level which is a biomarker for AD.

The results showed that treated AD with Lyc or/and WS can prevent increased level of $A\beta$ -42 in AD rats serum. The combination treated group showed the lowest level more than individual treated groups. The effect of Lyc on $A\beta$ -42 has not been studied. For WS, it was observed that daily treating with WS (1g/kg) for 30 days has improved memory decline and decreased $A\beta$ -42 level (Sehgala *et al.* 2012). This data showed the potential effect of Lyc and WS on reducing $A\beta$ -42 level which a harmful peptides in AD pathology, it is the most common type of $A\beta$ protein found in the brain and reducing its level will decrease $A\beta$ fibril formation, this give Lyc and WS a helpful benefit on treating AD.

In 1987, Hofstetter *et al.* discovered that Al^{+3} toxicity cause deficit in TH enzyme activity, they approved that daily administration of Al^{+3} (1.5 mg/animal) significantly decreased TH level and this was associated with a reduction in cholinergic enzymes. In our study, $AlCl_3$ significantly decreased TH levels in compared to normal group. In the brain of AD, reduction of TH is associated with neurological changes, decreased TH level indicates that there is a change in catecholamine levels which an important sign of AD. This finding in contract with Yabukiet *al.* (2014), who stated that TH level obviously decreased in AD mice model and this was associated with dopamine reduction.

In Lyc and/or WS treatments of $AlCl_3$ treated rats resulted in significant increase in TH level. Our finding in agreement with other studies on neuroprotection of Lyc and WS (Prakash *et al.* 2013; Jayaraj *et al.* 2019; Caleb *et al.* 2021), Lyc and WS have the ability to restore dopaminergic enzymes through induce TH activity. TH is a rate limiting enzyme in dopamine production reaction, any alteration in TH activity will result in dopaminergic neural loss in neurodegenerative diseases (Zhu, Zhang, and Zheng 2012). From this results we were able to demonstrate that Lyc and WS decreased $AlCl_3$ toxicity induced AD that lead to loss of TH neurons and this purpose the ability of them to improve dopaminergic neuron survival.

Na^+/K^+ ATPase enzyme is a marker for cell damage after toxic exposure. In the present study, Na^+/K^+ ATPase decreased after $AlCl_3$ administration, implying that $AlCl_3$ caused an intracellular accumulation of Na^+ resulting in cytotoxicity and cell damage. $AlCl_3$ can inhibit Na^+/K^+ ATPase activity by binding directly to thiol group at the active site of the enzyme preventing the thiol group from working in certain chemical processes (Silva and Gonçalves 2003). This result in agreement with previous studies (Elhadidy *et al.* 2018; Bulan *et al.* 2018).

The administration of Lyc or/and WS to the AD rats significantly increased the Na^+/K^+ ATPase activity in compared to AD group. This action may due to the presence of Huperzine A compound in Lyc. It has been investigated that Huperzine A isolated from Lyc prevent reduction in Na^+/K^+ ATPase in cell culture study (Gao and Tang 2006). It can be revealed that Lyc increased this enzyme concentration and protect against damage of neural cell. In WS treated group, Increased enzyme activity may be an action of WS on neural cell in a way for restoring the cell membrane ionic gradient. Over production of ROS can oxidize Na^+/K^+ ATPase enzyme, high oxidation in AD cause a reduction on Na^+/K^+ ATPase activity, the antioxidant property of WS protected and induced the enzyme activity (Jain and Mathur 2020).

As shown in result, the group that treated with both Lyc and WS possessed an overall higher preference scores as compared to separately plants treated groups. The above findings indicate the

potential cholinergic inhibitors, Anti-amyloidogenic and antioxidant effect that could be obtained from daily administration of Lyc and WS. The decreased levels of TH and Na⁺/K⁺ATPase in mixture treated groups may be due to the high free radical scavenging activity and enhancement of antioxidants provided when giving both treatments rather than giving separately. This finding indicates the effective treatment of Lyc and WS against AD induced by AlCl₃ in rats model.

Conclusion

From the present study, it could be concluded that Lyc and WS have a neuroprotective effect on AD rats model. They restored cholinergic system function by preventing AchE and BchE, which destroys Ach, reduced Aβ-42 level in serum, increased TH level in rats brain and the AD rat models. They also increased the Na⁺/K⁺ATPase activity which is a marker of cytotoxicity in AD neurons cells. The combination of both herbs could ameliorate the characteristics of AD more than treated separately. The findings of this study suggested the therapeutic potential of Lyc and WS in AD. Further clinical trials on AD patients are required to measure the efficacy of Lyc and WS on neurodegenerative diseases.

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