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AlCl₃ induced neuroinflammation and excitotoxicity in brain striatum mediated memory and motor dysfunction ameliorated via scopoletin enriched Canscora decussata extract

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ABSTRACT

Objective: Scopoletin is one of the dynamic and active constituents originate in Canscora decussata (CD) herb from Shankhpushpi family according to Indian system of medicine. This study aimed to characterize and to explore the role of scopoletin enriched CD (SECD) extract on AlCl₃-induced neuroinflammation and excitotoxicity in rat model. Methodology & Results: High-performance thin-layer chromatography finger printing of ethanol extract of CD confirmed that enriched scopoletin presence as its main constituent. AlCl₃ induced behavioural changes were reversed after SECD (200 and 400 mg/kg, i.p) and Rivastigmine (0.3 mg/kg, i.p) treatment by the way of Y maze, Rota rod, and grip strength experiments. SECD was investigated for anti acetylcholinesterase activity associated to memory and motor impairment. In addition to that, AlCl₃-induced inflammation and neurotoxicity was reversed significantly after SECD administration as it alleviates the rise of TNF α, IL-1β, and IL-6 release and the suppression of striatal glutamate and aspartate action. Further, our finding shows that AlCl₃-induced subcellular alterations include gliosis and lymphocytic infiltration in the cerebral cortex, as well as neuronal cell death, vacuolation, and cell membrane rupture in the hippocampus. Conclusion: This study concludes that treatment with SECD can be a novel phytochemical molecule for the retrieval of memory and motor impairment by preserving cholinergic and glutaminergic regulations, and to maintain the central morphological integrity.

Keywords: AlCl₃, aspartate, Canscora decussata, glutamate, inflammatory cytokines, scopoletin

INTRODUCTION

Neurotoxicity is defined as a change in nervous system function caused by exposure to specific chemical, biological, and physical agents that cause adverse effects such as neuronal cell degeneration.[1] Exposure to neurotoxins such as arsenic (As), lead (Pb), mercury (Hg), aluminum (Al), magnesium (Mg), and n-hexane which may lead to neurodegenerative effects causing neuronal cell lose; hence, these neurotoxins are toxic at very low doses.[2] Aluminum (Al) is the world’s third most prevalent metal, accounting for around 8% of the planet’s shell. [3] Aluminum-induced neurotoxicity has multiple mechanisms such as apoptotic cell death, impair neurotransmitter content and synaptic transmission, inhibition of voltage gated channels, and long-term potentiation, thus this provides effective evidence for multiple targeted approach for the treatment of neurotoxicity.[4] It has higher blood brain permeability which cross blood brain barrier (BBB) showing increase in concentration of Al in brain regions, it involves in the development of neurodegenerative disorders such as Alzheimer’s (AD), Parkinson’s (PD), and multiple sclerosis.[5] AlCl₃ is a cholinotoxic agent that alters chemicals in the brain; non-adrenergic and cholinergic systems. Acetyl choline esterase (AChE) is an enzyme involved in the metabolism of Ach that causes neurobehavioral changes in a memory model.[6] It also showed increase in pro-inflammatory cytokines that triggers
the formation of inducible nitric oxide synthase and produce large amount of NO and impair glutamate-Nitric oxide – cGMP pathway.[7] Al causes excitotoxicity due to an increase in glutamate and aspartate levels.[8] Al induced oxidative stress increase lipid peroxidation and also decrease in Glutathione levels, thus causing electron leakage, increase in the activity of mitochondria, and enhance electron chain reaction.[9]

*Canscora decussata* (CD) is known from its common name “shankhpushpi” in southern India belonging to the family of Gentianaceae. There are seven herbs of shankhusphi, namely, *Convolvulus pluricaulis* Choisy. (*Convolvulaceae*), *Convolvulus microphyllus* Sieber ex Spreng. (*Convolvulaceae*), *Evolvulus alsinooides* Linn. (*Convolvulaceae*), *Evolvulus nummularius* (*Convolvulaceae*), *Clitorea ternatea* Linn. (*Papilionaceae*), *Canscora diffusa* R. Br., and *CD Schult*. The entire plant is used medicinally especially for memory boosting. CD has therapeutic potential on CNS mainly AChE inhibition, hypertension, inflammation, hepatoprotection, and postmenopausal osteoporosis.[10,11] CD contains several xanthones, triterpenoid’s, flavonoids, coumarin derivatives, and loliolides.[12] Mangiferin (Xanthone glycoside) and Scopoletin (Phenolic coumarin) are the two major phytoconstituents present in CD confirmed by high-performance thin-layer chromatography (HPTLC)[13] and are important for CNS activity majorly antioxidant activity and memory enhancing activity.[14] Hence, the present study hypothesis that characterization of scopoletin from CD extract and whether the scopoletin enriched ethanolic extract of CD (SECD) have a therapeutic role on AlCl<sub>3</sub>-induced memory impairment and motor incoordination.

**MATERIALS AND METHODS**

**Preparation and Characterization of Extract by HPTLC Finger Printing**

Analytical grade, powdered CD was obtained from Kshipra biotech private limited, Indore. 200 mg CD powder was defatted, extracted with ethanol (99%) and suspended in 3% CMC. Precoated and preactivated HPTLC plates of silica gel 60 f<sub>254</sub> plated with aluminum of 10 × 10 cm with 250 μm thickness. Ethanolic extract of CD (1 g) was dissolved in 10 ml of methanol. Scopoletin of 1 mg/50 ml of methanol was prepared with concentration of 20 ng/μl. The standard was applied ranging from 1 to 5 μl, and the sample was 2 μl. CAMAG LINOMAT V an automatic sample application device, was used to apply the samples in the form of a band. Plates were prewashed in methanol and activated for 5 min at 60°C. A constant application rate of 0.1 μl/s with band width of 5 mm. The slit dimension was 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. The solvent system utilized was chloroform: toluene: methanol (8:1:1), and the plates were developed in a chamber saturated with the solvent system. After drying plates were scanned densitometrically at 325 nm using mercury lamp. The bands in extract were identified by comparing with the standard Scopoletin Rf of 0.27. Scopoletin content in ethanolic extract was found to be 107 ng/gm of extract.[13]

**Animals, Chemicals, and Study Protocol**

Male Wistar rats of average weight (120–180 g) and experimental protocol was accepted by the Institutional Animal Ethics Committee (IAEC) with proposal number: PSG Institute of Medical Sciences and Research (PSGIMS and R-358/2017/IAEC). Experiments were conducted according to CPCSEA guidelines. Hi-Media lab limited in India provided the aluminum chloride, acetylthiocholine iodide (ATCI), and 5,5'-dithiois [2-nitrobenzoic acid] (DTNB). Scopoletin, TNF-α, IL-1β, IL-6, Glutamate, and Aspartate standard from Sigma-Aldrich, USA, precoated HPTLC plates from Merck, Mumbai, India. All other chemicals, reagents, and solvents were of analytical grade unless mentioned. Animals were randomly assigned into five groups, containing six animals in each group. Group I- received 3%w/v Carboxymethyl cellulose (CMC), p.o.; Group II -AlCl<sub>3</sub>-100 mg/kg, i. p. for 42 consecutive days; Group III-AlCl<sub>3</sub> and Rivastigmine-0.3 mg/kg; Group IV -AlCl<sub>3</sub>-100 and SECD-200 mg/kg; and Group V - AlCl<sub>3</sub>-100 and SECD-400 mg/kg.

**Behavioral Assessment**

The Y Maze test is used to examine the novelty preferences of rodents. Rats were placed in the start arm of the apparatus with one arm closed, and given 5 min to explore the remaining two arms. In the test trial, rats were again placed in the start arm of the Y maze and permitted to roam freely for 5 min,
this time all the three arms were open. The “novel arm” was defined as the one that was closed during the sample test (previously unexplored). The total time spent, time to reach the novel arm and the number of arm entries was scored during the test trial.\textsuperscript{[15]}

The rotarod treadmill utilized to assess the motor performance. Two training sessions were conducted for 5 min at a rate of 25 rpm. The control animal was able to remain on the rod for about 180 s. The animal was placed and fall of time was noted.\textsuperscript{[16]} The neuromuscular activities of rodents were studied using a Bio-Seb grip strength meter to determine the maximal peak force generated. The rat was held by the tail and lowered towards the equipment, with the grip strength meter positioned horizontally. The animals are permitted to grasp the metal grid before being dragged backwards in the horizontal plane. The peak tension is defined as the force given to the grid right before it loses hold.\textsuperscript{[17]}

Biochemical Estimation

The brain AChE activity was measured using Ellman’s et al.\textsuperscript{[18]} 1961 method with slight modifications. Briefly, 20 mg of brain tissue/mL of phosphate buffer (0.1M; pH8) was homogenized and 0.4 mL aliquot of brain homogenate was added to a cuvette containing 2.6 mL of 0.1M phosphate buffer, 100 µL of DTNB reagent (10 mg DTNB in 100 mL of phosphate buffer, pH8.0). The substrate acetylthiocholine iodide 20 µL (75 mg of acetylcholine iodide per 50 mL of distilled water) was added and the optical absorbance was monitored every 2 min at 412 nm for 10 min. Protein content estimated by Lowry et al.\textsuperscript{[19]} 1951. Brain striatal glutamate and aspartate content were estimated by HPTLC (CAMAG — version 1.3.4, USA) according to Babu et al.\textsuperscript{[20]} Quantifications of brain pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) were assayed using manufacturer’s protocol of Duo Set Elisa Kit.

Histopathological Studies

Brains were isolated and preserved in 10% phosphate buffer formaldehyde before being embedded in paraffin and cut into 5 µm longitudinal pieces. Hematoxylin and eosin (H and E) were used to stain the sections, which were then inspected under a microscope.

Statistical Analysis

Data were presented as Mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests and for continuous assessment study, Two-way ANOVA followed by Bonferroni post hoc test performed. The obtained data were analyzed using GraphPad prism 5.03 version software.

RESULTS

HPTLC Finger Printing of Standard Scopoletin and SECD Extract

The existence of Scopoletin in ethanolic extract was confirmed by HPTLC with the similar Rf of 0.27 and the quantity of Scopoletin present in ethanolic extract was found to be 107 ng/gm of extract (Figure 4).

Effect of SECD on Y Maze Novelty Performance

The control group has a greater number of entries, spent greater time in novel arm and decrease in time taken to reach novel arm which indicates spatial recognition memory of the animal. AlCl\textsubscript{3} treated rats showed significant reduction $F_{(4, 20)} = 28.34$, ($P < 0.01$) in no. of entries in the novel arm, spent less time in novel arm $F_{(4, 20)} = 58.60$, ($P < 0.05$), significant rise $F_{(4, 20)} = 46.99$, ($P < 0.01$) in time required to reach the novel arm compared with the control group. Treatment with rivastigmine 0.3 mg/kg and SECD 400 mg/kg ($P < 0.01$), 200 mg/kg ($P < 0.05$) showed significant increase $F_{(4, 20)} = 28.34$, in the no. of entries in the novel arm, increase $F_{(4, 20)} = 58.60$ in the time spent in the novel arm, significant reduction $F_{(4, 20)} = 46.99$, ($P < 0.01$) in time required to reach the novel arm compared to AlCl\textsubscript{3} group [Figure 2a-c].

Effect of SECD on Rota Rod Test

The increase in falling time of rats indicates improved motor coordination. AlCl\textsubscript{3} treated rats showed significant neurological deficits, since fall off time was decreased on 21st and 42nd days when compared with control group $F_{(4, 20)} = 16.86$, ($P < 0.01$). Rivastigmine (0.3 mg/kg) treated rats resulted significant improvement $F_{(4, 20)} = 16.86$, ($P < 0.01$) in fall off time on 42nd day when compared to AlCl\textsubscript{3} treated rats. SECD (200 and 400 mg/kg) treated rats showed significant increase in fall off time $F_{(4, 20)} = 16.86$, ($P < 0.01$) on 42nd day as compared to AlCl\textsubscript{3} treated rats [Figure 2d].

Effect of SECD on Grip Strength

Figure 3a represents the effect of SECD on muscle strength in AlCl\textsubscript{3} treated rats. The increase in force applied to the grid indicates improved motor coordination and muscle strength. AlCl\textsubscript{3} treated rats showed significant neurological deficits when compared to control group $F_{(4, 20)} = 23.89$, ($P < 0.01$) as observed decreased muscle strength on 21st and 42nd days. Rivastigmine (0.3 mg/kg) treated rats resulted significant improvement $F_{(4, 20)} = 23.89$, ($P < 0.01$) in muscle strength on 42nd day in comparison to AlCl\textsubscript{3} treated rats. SECD (200 and 400 mg/kg) treated rats showed significant increase in muscle strength $F_{(4, 20)} = 23.89$, ($P < 0.01$) on 42nd day in comparison to AlCl\textsubscript{3} rats.

Effect of SECD on AChE Activity

Alteration in AChE activity may affect the cholinergic transmission process leading to impairment in learning/memory and motor coordination. AlCl\textsubscript{3} treated group resulted significant increase $F_{(4, 20)} = 252.1$, ($P < 0.01$) in the AChE activity as compared to control group. Meanwhile, Rivastigmine, SECD (200 and 400 mg/kg) resulted significant reduction $F_{(4, 20)} = 252.1$, ($P < 0.01$) in AChE activity when compared to AlCl\textsubscript{3} treated group [Figure 3b].

Effect of SECD on Excitatory Neurotransmitters in Rat Brain

Decrease in glutamate and aspartate levels in treatment groups when compared to AlCl\textsubscript{3} treated group indicates the alleviation...
of excitotoxicity in brain striatum [Figure 3c and d]. AlCl$_3$ treated rats exhibited significant increase $F_{(4, 20)} = 775.3$, ($P < 0.01$) in glutamate and $F_{(4, 20)} = 90.86$, ($P < 0.05$) in aspartate levels when compared to control group. Treatment with rivastigmine 0.3 mg/kg ($P < 0.01$), SECD 200 and 400 mg/kg ($P < 0.05$ and $P < 0.01$) showed significant decrease $F_{(4, 20)} = 775.3$, in glutamate and $F_{(4, 20)} = 90.86$ in aspartate levels in comparison to AlCl$_3$ treated group (Ref: Supplementary File 1 and Supplementary File 2).

**Effect of SECD on Pro-Inflammatory Cytokines**

Figure 5a-c The decrease in TNF$\alpha$, IL-1$\beta$, and IL-6 levels in treatment groups in comparison to AlCl$_3$ treated group indicates the mitigation of neuronal inflammation in brain. AlCl$_3$ treated rats resulted significant improvement $F_{(4, 20)} = 710.5$, ($P < 0.01$) in TNF$\alpha$, $F_{(4, 20)} = 2202$, ($P < 0.01$) in IL-1$\beta$, $F_{(4, 20)} = 4830$, ($P < 0.01$) in IL-6 levels when...
compared to control group. Treatment with Rivastigmine 0.3 mg/kg ($P < 0.01$), SECD 200 and 400 mg/kg ($P < 0.01$) resulted significant reduction $F_{(4, 20)} = 710.5$, ($P < 0.01$) in TNF$\alpha$, $F_{(4, 20)} = 2202$, ($P < 0.01$) in IL-1$\beta$, and $F_{(4, 20)} = 4830$, ($P < 0.01$) in IL-6 levels in comparison to AlCl$_3$ treated animals.

**DISCUSSION**

The main finding of this study is that treatment with Scopoletin-enriched CD extract reduces cognitive impairment and motor incoordination in AlCl$_3$-treated animals by lowering AChE activity. CD contains $\beta$ carotene, Rutin, scopoletin, and Mangiferin as main constituents. Astonishingly, CD has the highest AChE inhibitory activity of any shankpushpi herb. Besides that, CD has been shown to be an antioxidant with a direct effect on signaling mechanism to improve neural communication, improve neuroprotective adaptation, reduce stress signaling, modulate extracellular signaling for kinase activity, increased insulin like growth factor I, regulate MAPK significantly contribute to cognitive improvement. Scopoletin is a phenolic coumarin derivative reported to improve age treated rats showed scattered lymphocytic infiltration and gliosis, declining of neuronal cell loss, and no vacuolation.

**Morphological Changes in Prefrontal Cortex and Hippocampus**

Figure 6a and b Control rat showed no change in architect of Prefrontal cortex and Hippocampus, AlCl$_3$ 100 mg/kg rat showed diffused lymphocytic infiltration, reactive gliosis, vacuolation, and disruption of cell membrane, neuronal cell loss. Rivastigmine 0.3 mg/kg treated rat showed lymphocytic infiltration, less neuronal cell death, and no cell membrane rupture. SECD 200 mg/kg treated rats showed diffused lymphocytic infiltration and reactive gliosis, reduced disruption of cell membrane and neuronal cell loss. SECD 400 mg/kg treated rats showed scattered lymphocytic infiltration and gliosis, declining of neuronal cell loss, and no vacuolation.

Figure 4: (a) 3D chromatogram of glutamate and aspartate. (b) Chromatogram of standard glutamate. (c) Chromatogram of standard aspartate.

Figure 5: Effect of SECD on pro-inflammatory cytokines. (a) TNF$\alpha$, (b) IL-1$\beta$, (c) IL-6. The data are presented as a Mean ± SEM, ($n = 6$), One-way ANOVA was used for statistical analysis, followed by Tukey’s Multiple Comparison Test. a denotes $P > 0.01$ versus control. x, y denotes $P < 0.01$, $P < 0.05$ versus AlCl$_3$ treated group at respectively.
related cognitive decline, anti-inflammatory activity, antiproliferative, antioxidant, and radical scavenger activity a therapeutic for neurodegenerative disease. It has agonistic effects on Nicotinic acetylcholine receptors (nAChRs), enhances N-methyl-D-aspartate receptor (NMDA) dependent Long-term potentiation (LTP) in hippocampus. Furthermore, scopoletin increases the presynaptic Ach release, showing cognitive improving properties. \[21\]

The mechanism of aluminum toxicity is extremely crucial as it has been linked to a number of neurodegenerative diseases, including oxidative brain damage and the generation of reactive oxygen species, altered neurotransmitter biosynthesis, inflammatory reactions (gliosis), reduced utilization of glucose, changes in the rate of transmembrane diffusion and the BBB transport system (BBB). \[26,27\] Aluminum hampered retrieval and acquisition of spatial recognition memory in the Y-maze, according to our findings. Toxicity-induced rats showed a decrease in the number of entries and less time spent in the novel arm, as well as an increase in the time it took to get to the novel arm; this reflects the impairment in spatial memory. Treatment with SECD resulted in a better protective response and an improvement in an animal’s behavioral state, as it antagonized the spatial memory deficits caused by aluminum. After AlCl3 treatment, muscle and locomotion activities were lowered; aluminum levels above a certain threshold not only affect memory and yet also motor functions by reducing grip strength. \[28\] In this study, AlCl3 caused a considerable decrease in motor coordination, tested by rotarod and grip strength showing changes in endurance, balance and muscle strength, in reference with increased AChE levels in the homogenates of rat brain. \[29,31\] This was reversed after SECD treatment, showed increase in duration of sustained balance on rotarod indicating improved motor coordination and increase in force applied on grip strength indicating improved muscle tone. In general, aluminum disrupts cholinergic neurotransmission, impairing cognitive behavior and reducing motor function. Elevated AChE causes Al to have a neurotoxic effect, resulting in a decrease in Acetylcholine (Ach) synthesis. \[31-35\] In accordance with the preceding statement, AlCl3 increased the AChE enzyme activity that catalyses the hydrolysis of Ach in this study, where immediate clearing of Ach at the synapse leads to impairment in muscle function and cognitive functions. \[6\] Meanwhile, SECD significantly reduced the AChE activity in aluminum-treated rats brains indicating increase in the release of Ach in synapse. These finding indicated that ethanolic extract of SECD significantly improved the memory and motor coordination. AlCl3 build up in the brain can disrupt the neuronal signal transduction system that is linked to glutamate receptor that can causes excitotoxicity in neuronal cells by altering glutamine synthase and availability of glutamate. \[27\] In this study, there was a considerable increase in glutamate concentration in brain region might be due to deposition of aluminum that enhances adenylate cyclase induced cAMP activity in brain. Rivastigmine and SECD effectively decreased the glutamate and aspartate levels by attenuating the Al-glutamate complex due to increase in glutamate decarboxylase release in neuronal cells. Furthermore, AlCl3 increased the protein content of tumor necrosis factor (TNF), interleukins (IL-6 and IL-1) in the brain striatum, which could be attributed to astrocyte and microglia activation. ROS can also cause transcription factors to be activated such as nuclear factor kappa B (NF-kB), p53 in glial cells which produces inflammatory cytokines and excitotoxins. \[36,39\] IL-6 and TNFα are the prime contributors to motor in-coordination after AlCl3 exposure, causing loss of muscle strength. Decreased cytokine gene expression in the brain is necessary for skillful motor behavior \[40,41\] whereas, rivastigmine and SECD showed significant decrease in inflammatory cytokines level indicating improvement in

<table>
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<tr>
<th>Control</th>
<th>AlCl3 100mg/kg</th>
<th>Rivastigmine 0.3mg/kg</th>
<th>SECD 200 mg/kg</th>
<th>SECD 400mg/kg</th>
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**Figure 6:** (a) Morphological changes in prefrontal cortex. (b) Hippocampus
memory and motor co-ordination. According to studies, the aluminum-induced morphological changes are cellular infiltration (lymphocytic), neuronal loss, cytoplasmic vacuolation, chromatin condensation, ghost cells in the cerebral cortex, edema, hemorrhage, and gliosis. Our findings show that subcellular alterations in ACl, include gliosis and lymphocytic infiltration in the cerebral cortex, as well as neuronal cell death, vacuolation, and cell membrane rupture in the hippocampus. There was less lymphocytic infiltration and no gliosis in the cerebral cortex after treatment with SECD, as well as less neuronal cell death and no vacuolation. Matter of fact, the presence of Scopoletin in CD can be linked to improved memory and motor coordination activity. This herb’s synergistic activity could be attributed to the presence of xanthone derivatives.

CONCLUSION

According to the study findings, a Scopoletin-enriched ethanolic extract of CD improved ACl3-induced memory impairment and motor in-coordination. Scopoletin, a phytochemical found in an ethanolic extract of CD, may be responsible for memory retention and motor coordination. As a result, this herb has the potential to be used as a neuroprotective agent in aluminum-induced neurotoxicity.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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