ABSTRACT

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An experimental approach to unearth the immunomodulatory aspects of *Withania somnifera* towards altered physiology and Hedgehog signalling in the bone marrow of leukemic mouse

Submitted by

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N-nitroso compounds (NOC) are potent DNA alkylating agent having serious carcinogenic effects on biological system. Environmental exposure to NOC can impart genomic insults to the bone marrow cells leading to the malignant transformation of hematopoietic stem/ progenitor population as well as to the haematopoiesis supportive microenvironmental components resulting in the development of leukaemia. In this study, we would like to unearth the detrimental consequences of NOC-induced leukaemia and the ameliorating effects of Ashwagandha and its isolated counterpart, Withanolide D, on the dreadful disease condition.

Four groups of animals were taken for experimental purposes viz; group I= Ethyle nitroso urea (ENU) mediated leukemic mice (L); group II=control mice (C); group III = leukemic mice treated Ashwagandha (L+A); and group IV =leukemic mice treated with extracted Withanolide D, (L+WD). Group III and IV received Ashwagandha and isolated Withanolide D respectively via oral route, and the other two groups received an equal volume of distilled water. Various physiological, haematological, cytological, immunofluorescence, and flow cytometric studies were taken into consideration before and after the administration of Ashwagandha and Withanolide D. Various doses of Ashwagandha and Withanolide D were administered to evaluate the LD_{50} and EC_{50} values. Estimated LD_{50} and EC_{50} values of Ashwagandha were 1175 mg/kg bodyweight and 600mg/kg bodyweight, respectively, whereas the LD_{50} and EC_{50} values of Withanolide D were 75 mg/kg bodyweight and 37.5 mg/kg bodyweight, respectively.

A significant decline in locomotor activity and bodyweight was noted due to the leukemic insult caused by ENU. Haematological profiling depicted an increase in WBC count, reticulocyte count, abnormal blasts, and other parameters indicative of leukemic pathophysiology. The reflection of the disease condition was further noted in the cytological analysis. Excessive cellular proliferation, the presence of abnormal cells, and significantly fewer dead cells were key indicators. Upon further investigation, various cellular signalling pathways and key cytological processes were found to be deregulated. Components of the hedgehog signalling pathway, viz; SHH, PTCH, SMO, and GLI1, were over-expressed, while expressional decline was observed in case of HHIP, SUFU, and GLI3. Overexpression of GSK3β and β-TrCP was found to be associated with the activation of the NRF2/KEAP1 axis. Simultaneously, expressional increase of key cytoskeletal proteins, viz; β actin,

β tubulin, N-cadherin, and vimentin, was also noted. Over expression of RNAi machinery components viz, DICER, DROSHA, AGO1, and AGO2, was found to be associated with the malignant transformation of bone marrow during leukaemia. LIN28, a key component modulating many cellular functions was also found to be up-regulated in diseased condition, which can be correlated with the over-expression of N-MYC oncogene. Molecular components associated with autophagy viz; P62 and ATG12, were also found to be downregulated which might have aided in the accumulation of defective proteins as well as diminished the DNA repair mechanism. Elevated levels of BCL2, TERT, and decreased expression of PUMA, Caspase 3, CD11b, and CD95 can be correlated with the malignancy associated anti-apoptotic phenomenon. Thus, a wide range of molecular expressional alterations was found to be associated with the ENU mediated leukemic transformation of the bone marrow.

With the administration of Ashwagandha and the isolated Withanolide-D, body weight and locomotory activity were found to be shifted towards normalcy in the treated. The confirmation of the positive effects of Ashwagandha and Withanolide D was also noted in the haematological as well as in case of cytological profiling. Expressions of Hedgehog signalling components were found to be shifted towards their normal values. The NRF2/KEAP1 signalling axis was also found to be modulated, which imparted a co-modulatory effect on the components of the hedgehog signalling cascade, viz; SHH, SUFU, GLI1, etc., with a shift towards normal condition. Expressions of cytoskeletal proteins in the phytochemical treated groups were also found to be shifted towards normal values which can be correlated with the disruption of spindle structure, microtubular pathways, and protein-protein interactions in the treated leukemic cells resulting in checking of abnormal proliferation and migration. Simultaneously, expressions of RNAi components as well as that of tumorigenic N-MYC and BMI-1 were also found to be declined significantly with the effect of Ashwagandha and Withanolide-D. Expressional restoration of autophagic machineries viz; P62 and ATG12 were also noted. Malignancy related anti-apoptotic phenomenon also got reversed by the effects of the phytochemicals as shown by the expressional alterations of TERT, BCL2, Caspase 3, CD11b, CD95, and PUMA.

Taken together all the findings, it appears that, Ashwagandha as well as the extracted active compound- Withanolide D showed significant anti-leukemic properties by modulating physiological, haematological, cytological, cytoarchitectural, protein processing and trafficking molecular aspects, balancing the dynamics of autophagy, proliferation and apoptosis together with the associated expressional alterations of the components of hedgehog signalling cascade in NOC-induced leukaemia, with a promise to open new avenues in the therapeutic regimen for the dreadful disease concerned.

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