Pharmacognosy Magazine
ISSN: 0973-1296

PHCOG MAG.
An official Publication of Phcog.Net

PHCOG MAG.: Research Article

Antimutagenic activity of Ashwagandha against lead nitrate: A genetic and non-genetic biomarkers' evaluation

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ABSTRACT

The genetic and non-genetic biomarkers were evaluated to study the protective effect of methanolic extract of ashwagandha (125, 250 and 500 mg/kg b.w p.o) against Lead nitrate (40 mg/kg b.w i.p) induced toxicity. Frequency of micronuclei (MN) was evaluated as genetic marker using mouse bone marrow micronucleus test in mice treated with Ashwagandha and lead nitrate. The liver enzymes SOD, catalase and GSH were estimated as non-genetic biomarkers in ashwagandha and lead nitrate treated animals. A dose dependent and time related inhibitory effect of ashwagandha treatment on frequencies of MN induced by lead nitrate in both polychromatic (PCE) and normochromatic (NCE) cells MN was observed. The decrease in P/N ratio induced by lead was also prevented by the ashwagandha treatment. The level of SOD, catalase and GSH in liver was significantly depressed following lead nitrate administration and the ashwagandha treatment enhanced the level of these enzymes significantly. These findings suggest that ashwagandha extract is effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of reactive oxygen radicals generated by lead nitrate.

Key Words: Antimutagens, Antioxidants, Ashwagandha, Mutagen.

INTRODUCTION

Environmental carcinogens and mutagens pose a major health risk. Untoward mutations are associated with a number of serious diseases like cancer, aging, arthritis, cardiovascular diseases and infectious diseases. There are numerous recognized sources of chemical mutagens and carcinogens in complex mixtures, including metals, cigarette smoke, insecticides, and constituents of industrial waste and urban pollution. Diverse environmental, industrial, dietary and natural chemicals are capable of inducing genotoxic effects in man. Many of the pollutants released into the atmosphere and water, the residues of pesticides and toxins present in the foods and drugs, are common agents of mutagenic damage in human population. Lead has been recognized as an ubiquitous environmental pollutant (1). It is known to produce reactive oxygen species resulting in DNA damage and lipid peroxidation (2). Liver and kidney are the major target organs of lead toxicity. Until now the studies regarding regulation of lead toxicity are restricted to some chelating agents (3) and few antioxidants such as vitamin C and vitamin E (4-5).

Phytochemicals are capable of preventing the process of mutagenic damage at one or more of the stages. They play a variety of roles such as antioxidants, suppressors of tumor growth, cytoprotectives, enzyme modulators, chemical inactivators, and free radical scavengers. *W. somnifera* Dunal, family Solanaceae; commonly known as ashwagandha, a subtropical under shrub is known to have several medicinal properties such as sedative, hypotensive, analgesic, anti-aging, aphrodisiac, anti-inflammatory, anti-tumour, radiosensitising and many others (6-8). Recently the importance of *W. somnifera* root extract in the regulation of lead toxicity with special reference to lipid peroxidation process has been investigated in the liver and kidney tissue of mice (2).

Among the techniques to detect genetic and genotoxic effects, the micronucleus (MN) test is often used as genetic marker and level of antioxidant enzymes as non-genetic markers. The present investigation attempts to reveal the efficacy of root extract of *W.somnifera* to inhibit the lead induced damage using these markers.

Materials and Method

Preparation of extract:

Ashwagandha roots were collected, authenticated, air-dried and powdered. The powdered drug was defatted using petroleum ether and refluxed with methanol for four hours. The residue was further extracted again with methanol for two hours. The extracts were then pooled together and evaporated to dryness using rotary vacuum evaporator.

Micronucleus test

Experimental protocol suggested by Hyashi et al (9) was adopted for micronucleus test. The protocol is in accordance with OECD (Organization of Environmental Carcinogen Detection) and WHO guidelines for mutagenicity studies.

Animals

Swiss albino laboratory bred mice weighing 25±2 g maintained in standard laboratory conditions were used. Animals were housed in groups of six in polypropylene cages and maintained in controlled temperature (27±2^{0}C) and light cycle (12 h light and 12 h dark). They were fed with Amrut Laboratory Animal Feed (Nav

Maharashtra Chakan oil Mills Ltd, Pune). Water was supplied *ad libitum*. Ethical clearance for the use of animals was obtained from Institutional Animal Ethics Committee of Al Ameen college of Pharmacy. The animals were divided in eight groups of 18 animals each.

Treatment

Group I served as control. The animals were kept on normal diet and were administered 0.2 ml of water by oral route for 7 days. The Group II received the challenge as 40 mg/kg b.w of lead nitrate by i.p. route. The groups III and IV received methanolic extract of ashwagandha (125 mg/Kg) by oral route for seven consecutive days. The groups V and VI received methanolic extract of ashwagandha (250 mg/Kg) by oral route for seven consecutive days. The groups VII and VIII received methanolic extract of ashwagandha (500 mg/Kg) by oral route for seven consecutive days. After half an hour of the last dose of ashwagandha the animals of group IV, VI and VIII received the lead nitrate challenge.

Extraction of bone marrow

From each group, 6 animals were sampled and sacrificed by cervical dislocation at 24, 48 and 72 hrs after the last treatment. The animals were dissected, femur and tibia were removed and the bone marrow was flushed out with 5% bovine serum albumin into a cavity block. A suspension was made and centrifuged. The supernatant was drawn off and the marrow was used for preparation of smear.

Staining of slides

The smear was made on clean slides and fixed in absolute methanol. The slides were then stained with May grunwalds stain diluted with phosphate buffer (6.8) and Giemsa stain freshly diluted with phosphate buffer for 15 and 10 minutes respectively. The slides were washed with buffered water and allowed to stand undisturbed for differentiation. They were then dried and scanned for presence of micronuclei in polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). 1000 PCEs and corresponding NCEs per animal were counted to calculate PCE/NCE (P/N) ratio.

Estimation of antioxidant enzymes in liver

Liver of the animals of all the groups were collected and cleaned in buffered saline (pH 7.4). 5% liver homogenates were prepared using 0.25 % sucrose in phosphate buffer and 0.5% potassium chloride in phosphate buffer. The homogenates were centrifuged at 500-x g for 10 min and the supernatant was used for estimation of proteins, SOD, catalase and GSH.

Estimation of proteins: Standard Folin Cio Calteu method was used to estimate the amount of proteins (10).

Estimation of enzymes: Amount of SOD was estimated using method described by Beauchamp and Fridovich (11). The amount of catalase was estimated using method described by Aebi (12) .The amount of glutathione (GSH) was estimated using method described by Tietze (13).

Data analysis

Results were expressed as mean ± SEM. The results were subjected to statistical analysis using unpaired't' test. Data were considered significant at $p < 0.05$.

Results

P/N ratio, percentage of MN and level of enzymes in control groups: P/N ratio and % frequency of MN in PCEs and NCEs were determined in control groups of animals. The results (Table 1, 2 and 3) indicated that the P/N ratio as well as percentage of micronuclei in PCEs and NCEs in bone marrow is within the stated limit (14). The level of SOD, catalase and GSH were also found to be within the limits stated in the other studies.

Effect of ashwagandha treatment: There was no significant increase in the % frequency of MN in PCEs and NCEs in ashwagandha treated animals at all the doses tested. The variation in the P/N ratio was also statistically insignificant when compared to control group. These observations indicate that ashwagandha *per se* lacks any mutagenic potential at the doses tested. (Table 1, 2 and 3). However there was an increase in the level of SOD, catalase and GSH enzymes in animals treated with ashwagandha extract compared to control group (Fig 1, 2 and 3)

Effect of lead nitrate treatment: The lead nitrate was proved to be a potent mutagen. There was a decrease in P/N ratio after the lead nitrate challenge at 24 hrs ($p<0.001$), 48 hrs ($p<0.01$) and 72 hrs ($p<0.05$). There was a significant increase in frequency of MN in both PCEs ($p<0.001$) and NCEs ($p<0.001$) after the challenge at all the tested intervals (Table 1,2 and 3). The level of SOD, catalase and GSH was significantly depressed ($p<0.001$) by lead nitrate treatment (Fig 1,2 and 3).

Antimutagenic activity of Ashwagandha: The ashwagandha treatment showed a significant antimutagenic activity against lead nitrate induced mutagenic damage. The decrease in P/N ratio induced by lead nitrate was prevented by all the treatments at all the tested intervals. There was a significant reversal ($p<0.001$) of increase in frequency of micronuclei in both PCEs and NCEs after all the three intervals (Table 2 and 3) at all the doses tested. The decrease in level of SOD, catalase and GSH induced by lead treatment (Fig 1,2 and 3) was also significantly reversed ($p<0.001$).

Discussion

Role of various plant extracts as antimutagens is being increasingly recognized (15). These extracts act directly on mutagens or on those agents that inhibit the generation of active mutagenic forms (16). We investigated the antimutagenic activity of ashwagandha: the inhibitory effect of ashwagandha against formation of micronuclei induced by lead nitrate in bone marrow cells of mice.

Micronuclei are chromatid/chromosome fragments that are left behind after expulsion of the main nucleus

during maturation of erythroblasts to erythrocytes in the bone marrow. These represent the consequence of DNA damage caused by externally administered substances.

In the present study the genotoxicity of lead nitrate was evident in the multisampling micronucleus test. Administration of lead nitrate (40 mg/kg b.w) caused a significant increase in percentage frequency of micronuclei. It has been reported that lead induced mutations may not be a result of direct damage to DNA but may occur via indirect mechanisms including disturbances in enzyme functions important in DNA synthesis and/or repair (17). Another study has reported oxidative damage produced by lead in mice (18). In our study the level of antioxidant enzymes, SOD, catalase and GSH were significantly depressed by lead nitrate treatment. Hence DNA damage by lead may be due to depletion of antioxidant enzymes.

Table 1: Effect of lead nitrate and ashwagandha on P/N ratio

Treatment	24 hrs	48 hrs	72 hrs
Control	1.01±0.05	1.01±0.05	1.01±0.05
LN	0.70±0.03***	0.66±0.11**	0.68±0.11**
WS (125mg)	1.00±0.01	1.00±0.01	1.00±0.01
WS (250mg)	1.01± 0.02	1.00±0.01	1.00±0.02
WS (500mg)	1.01±0.02	1.01±0.02	1.01±0.02
LN+WS (125mg)	0.82±0.08#	0.83±0.10##	0.89±0.02##
LN+WS (250mg)	0.84±0.06#	0.87±0.05##	0.92±0.01##
LN+WS (500mg)	0.86±0.05#	0.90±0.06##	0.94±0.10##

Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
** Control vs. LN treated group **-- p<0.01,*** --p<0.001*
 # LN vs. LN + WS #--p<0.05, ##--p<0.01, ###--p<0.001
LN-lead nitrate WS-Methanolic extract of W. somnifera

Table 2: Effect of lead nitrate and ashwagandha on % frequency of micronuclei in PCEs after 24,48 and 72 hrs

Treatment	24 hrs	48 hrs	72 hrs
Control	0.39±0.04	0.39±0.04	0.39±0.04
LN	1.74±0.20***	2.29±0.28***	3.00±0.31***
WS (125mg)	0.53±0.13	0.55±0.22	0.58±0.26
WS (250mg)	0.63±0.12	0.45±0.21	0.48±0.26
WS (500mg)	0.48±0.14	0.53±0.23	0.56±0.22
LN+WS (125mg)	0.98±0.11###	1.13±0.05###	1.08±0.21###
LN+WS (250mg)	0.84±0.10###	0.91±0.08###	1.04±0.22###
LN+WS (500mg)	0.74±0.08###	0.71±0.06##	0.64±0.12###

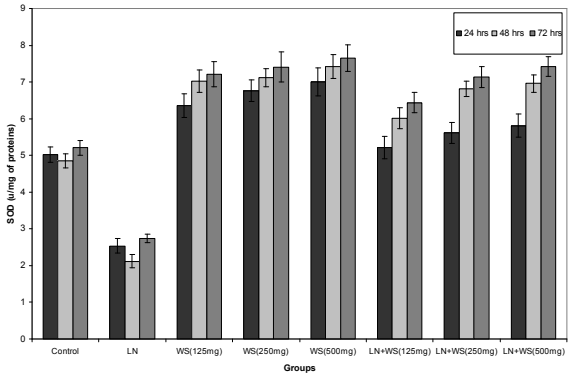
Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
** Control vs. LN treated group *** --p<0.001# LN vs. LN + WS ###--p<0.001*
LN-Lead nitrate WS-Methanolic extract of W. somnifera

Table 3: Effect of lead nitrate and ashwagandha on % frequency of micronuclei in NCEs after 24, 48and 72 hrs

Treatment	24 hrs	48 hrs	72 hrs
Control	0.20±0.02	0.20±0.02	0.20±0.02
LN	1.10±0.11***	1.14±0.04***	1.28±0.14***
WS (125mg)	0.23±0.04	0.33±0.02	0.23±0.01
WS (250mg)	0.13±0.10	0.23±0.04	0.20±0.02
WS (500mg)	0.12±0.01	0.22±0.01	0.20-±0.02
LN+WS (125mg)	0.56±0.21##	0.52±0.22###	0.52±0.21###
LN+WS (250mg)	0.46±0.21###	0.48±0.22###	0.50±0.21###
LN+WS (500mg)	0.36±0.21###	0.42±0.22###	0.42±0.21###

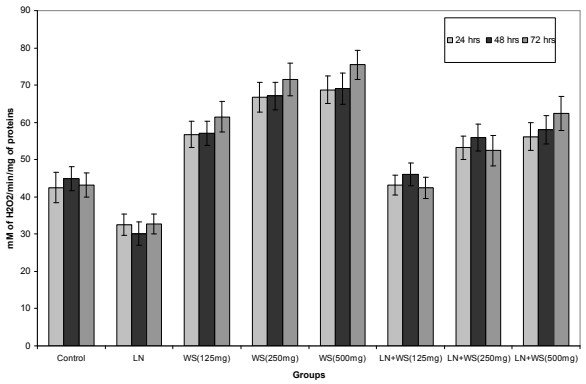
Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
** Control vs. LN treated group *** --p<0.001*
 # LN vs. LN + WS ##--p<0.01, ###--p<0.001
LN- Lead nitrate WS-Methanolic extract of W. somnifera

Fig : 1- Effect of LN and Ashwagandha on SOD



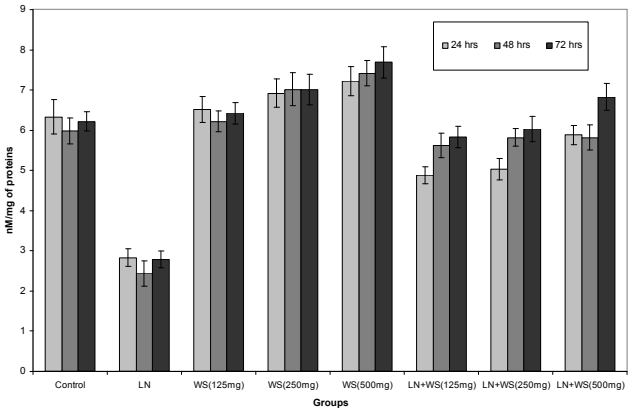
LN-Lead nitrate
WS-Methanolic extract of *W. somnifera*

Fig 2: Effect of LN and Ashwagandha on Catalase



LN-Lead nitrate
WS-Methanolic extract of *W. somnifera*

Fig 3 : Effect of LN and Ashwagandha on GSH



LN-Lead nitrate, WS-Methanolic extract of *W. somnifera*

The ashwagandha treatment exhibited dose dependent protective effect on the damage caused by lead nitrate. Another study has reported that ashwagandha is able to scavenge the oxidative damage produced due to lead toxicity (2). In our study there was a significant enhancement in level of SOD, catalase and GSH, in ashwagandha treated animals. Ashwagandha is a well-known antioxidant and is reported to cause an increase in anti oxidant enzymes in liver and brain of treated animals (2,18, 19). The level of these enzymes was significantly depressed by lead nitrate treatment and the ashwagandha treatment enhanced levels of these enzymes significantly. These findings suggest that ashwagandha extract is effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of active oxygen radicals generated in reactions initiated by the mutagens. Earlier studies with known antioxidants also showed that they help in counteracting lead-induced damage (4, 20).

CONCLUSION

Oxidative DNA damage has been implicated in mutagenesis and carcinogenesis. Naturally occurring antioxidants have been extensively studied for their capacity to protect organisms and cells from oxidative damage. In the present work, ashwagandha was indicated as an antimutagen against lead nitrate. The present investigation revealed the antigenotoxic potential of ashwagandha and hence it could prove to be an effective cytoprotective agent and provide protection against the deleterious implications of environmental or endogenous mutagens such as lead. However, investigations extending to other common environmental toxicants are needed. The use of ashwagandha can then be prescribed to minimise the risk caused by mutagenic agents, since exposure to several environmental pollutants cannot be completely eliminated.

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