International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 6(3): May-June 2016

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Life Sciences

Original Article……!!!

Received: 13-02-2016; Revised: 07-05-2016; Accepted: 12-05-2016

PRELIMINARY REPORT ON GENOTOXICITY STUDY OF ASHWAGANDHA (*WITHANIA SOMNIFERA*) IN VIVO IN MICE AND CYTOTOXICITY STUDY ON WRL68 CELL LINE

Trivikram M. Deshpande*, Sushama R. Chaphalkar

1. Scientist, Animal Biotechnology, VidyaPratishthan's School of Biotechnology (VSBT) Vidyanagari Baramati 413 133, Pune, India.
2. Director, VidyaPratishthan's School of Biotechnology, Vidyanagari Baramati 413 133, Pune, India.

Keywords:

Ashwagandha (*Withania somnifera*), root extract, Thin Layer Chromatography, Metaphase chromosomes , sperm morphology, WRL-68 cell line

For Correspondence:

Dr. Trivikram M. Deshpande

Scientist, Animal Biotechnology, VidyaPratishthan's School of Biotechnology (VSBT) Vidyanagari Baramati 413 133, Pune, India

E-mail:

tridesh@gmail.com

ABSTRACT

Imidazoles are an important class of heterocycles and include many substances of both biological and chemical interest. Insertion of the imidazole nucleus is an important synthetic strategy in drug discovery. Imidazole drugs have broad applications in many areas of clinical medicine. These are currently used as tools in pharmacological studies. The important therapeutic properties of imidazole related drugs have encouraged the medicinal chemists to synthesize and test a large number of novel molecules. In this investigation, it was of interest to synthesize imidazole by refluxing 9, 10-phenenthraquinone with aryl aldehyde, primary amines and ammonium acetate in the presence of glacial acetic acid and a novel series of imidazole derivatives. The structures of the compounds have been established on the basis of spectral analytical data. All the derivatives have been screened for their antimicrobial activities at the 100µg/ml and 200µg/ml against Candida albicans.

INTRODUCTION

Withania somnifera, also known as ashwagandha, Indian ginseng and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia. Clinical trials and animal research support the use of ashwagandha for anxiety, cognitive and neurological disorders, inflammation and Parkinson's disease. Ashwagandha's chemopreventive properties make it a potentially useful adjunct for patients undergoing radiation and chemotherapy. Ashwagandha is also used therapeutically as an adaptogen for patients with nervous exhaustion, insomnia and debility due to stress and as an immune stimulant in patients with low white blood cell counts (www.altmedrev.com/publications/9/2/211.pdf).

DESCRIPTION

Ashwagandha is a small, woody shrub in the Solanaceae family that grows about two feet in height. It can be found growing in Africa, the Mediterranean and India. As a result of this wide growing range, there are considerable morphological and chemotypical variations in terms of local species. However, the primary alkaloids of both the wild and the cultivated species appear to be the same. The roots are the main portion of the plant used therapeutically. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring. (www.altmedrev.com/publications/9/2/211.pdf).

Active Constituents

The major biochemical constituents of ashwagandha root are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides. At present, 12 alkaloids, 35 withanolides and several sitoindosides from this plant have been isolated and studied. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. Much of ashwagandha's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D (www.altmedrev.com/publications/9/2/211.pdf).

Chemistry

Since many of ashwagandha’s uses have not been scientifically validated, skepticism can naturally be expected when presented with a herb purportedly useful in so many ailments. In Ayurvedic medicine, there is a class of herbs, including WS, known as adaptogens or vitalizers. Adaptogens cause adaptive reactions to disease, are useful in many unrelated illnesses and

appear to produce a state of nonspecific increased resistance (SNIR) to adverse effects of physical, chemical and biological agents. They are relatively innocuous, have no known specific mechanism of action, normalize pathological effects and are usually glycosides or alkaloids of a plant. The chemistry of WS has been extensively studied and over 35 chemical constituents have been identified, extracted and isolated. The biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII) and withanolides with a glucose at carbon 27 (sitoindoside IX and X). WS is also rich in iron (http://www.altmedrev.com/publications/5/4/334.pdf).

[Chemical structure of withferin A and chemical structures of sitoindoside IX and X (http://www.altmedrev.com/publications/5/4/334.pdf).(text checked from website. now no checking)

Chemical structure of withferin A Chemical structures of sitoindosides IX and X

Mechanisms of action

The withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. Ashwagandha is thought to be amphoteric; i.e. it can help regulate important physiologic processes. The theory is that when there is an excess of a certain hormone, the plant based hormone precursor occupies cell membrane receptor sites so the actual hormone cannot attach and exert its effect. If the hormone level is low, the plant-based hormone exerts a small effect. Ashwagandha is also considered to be an adaptogen, facilitating the ability to withstand stressors and has antioxidant properties as well. Other studies have shown ashwagandha to have an immunostimulatory effect (www.altmedrev.com/publications/9/2.pdf).

Clinical Indications

Anti-Aging

In a double-blind clinical trial, ashwagandha was tested in a group of 101 healthy males, 50-59 years old, at a dosage of 3 grams daily for one year. A significant improvement in hemoglobin, red blood cell count, hair melanin and seated stature was observed. Serum cholesterol decreased and nail calcium was preserved. Erythrocyte sedimentation rate decreased significantly and 71.4 percent reported improvement in sexual performance (www.altmedrev.com/publications/9/2pdf).

Immunomodulation and Hematopoiesis:

A series of animal studies show ashwagandha to have profound effects on the hematopoietic system, acting as an immunoregulator and a chemoprotective agent. In a mouse study, administration of a powdered root extract from ashwagandha was found to enhance total white blood cell count. In addition, this extract inhibited delayed-type hypersensitivity reactions and enhanced phagocytic activity of macrophages when compared to a control group. Recent research suggests a possible mechanism behind the increased cytotoxic effect of macrophages exposed to *W. somnifera* extracts. Nitric oxide has been determined to have a significant effect on macrophage cytotoxicity against microorganisms and tumor cells. Iuvone et al demonstrated *Withania somnifera* increased NO production in mouse macrophages in a concentration-dependent manner. This effect was attributed to increased production of inducible nitric oxide synthase, an enzyme generated in response to inflammatory mediators and known to inhibit the growth of many pathogens (www.altmedrev.com/publications/9/2/211.pdf).

Ashwagandha exhibited stimulatory effects, both in vitro and in vivo, on the generation of cytotoxic T lymphocytes and demonstrated the potential to reduce tumor growth. The chemopreventive effect was demonstrated in a study of ashwagandha root extract on induced skin cancer in Swiss albino mice given ashwagandha before and during exposure to the skin cancer-causing agent 7,12-dimethylbenz[a]anthracene. A significant decrease in incidence and average number of skin lesions was demonstrated compared to the control group. Additionally, levels of reduced glutathione, superoxide dismutase, catalase and glutathione peroxidase in the exposed tissue returned to near normal values following administration of the extract. The chemopreventive activity is thought to be due in part to the antioxidant/free radical scavenging activity of the extract (www.altmedrev.com/publications/9/2/211.pdf).

An in vitro study showed withanolides from *Withania somnifera* inhibited growth in human breast, central nervous system, lung and colon cancer cell lines comparable to doxorubicin. Withaferin A more effectively inhibited growth of breast and colon cancer cell lines than did doxorubicin. These results suggest *Withania somnifera* extracts may prevent or inhibit tumor growth in cancer patients and suggest a potential for development of new chemotherapeutic agents (www.altmedrev.com/publications/9/2/211.pdf).

Anxiety and Depression:

In an animal study assessing the anxiolytic and antidepressive actions of ashwagandha compared to commonly prescribed pharmaceuticals, an extract of the root was administered orally to rats once daily for five days. The results were compared to a group administered the benzodiazepine lorazepam for anxiolytic activity and the tricyclic antidepressant imipramine for antidepressant investigation. Both the ashwagandha group and the lorazepam group demonstrated reduced brain levels of a marker of clinical anxiety. Ashwagandha also exhibited an antidepressant effect comparable to that induced by imipramine in the forced swim-induced "behavioral despair" and "learned helplessness" tests. Other similar studies confirm these results, lending support to the use of ashwagandha as an antistress adaptogen (www.altmedrev.com/publications/9/2/211.pdf).

Chronic Stress:

Chronic stress (CS) can result in a number of adverse physiologic conditions including cognitive deficit, immunosuppression, sexual dysfunction, gastric ulceration, irregularities in glucose homeostasis and changes in plasma corticosterone levels. In a rat model of chronic stress *Withania somnifera* and *Panax ginseng* extracts were compared for their ability to attenuate some effects of chronic stress. Both botanicals were able to decrease the number and severity of CS-induced ulcers, reverse CS-induced inhibition of male sexual behavior and inhibit the adverse effects of CS on retention of learned tasks. Both botanicals also reversed CS-induced immunosuppression but only the Withania extract increased peritoneal macrophage activity in the rats. The activity of the Withania extract was approximately equal to the activity of the *Panax ginseng* extract. *Withania somnifera*, however, has an advantage over Panax ginseng in that it does not appear to result in ginseng-abuse syndrome, a condition characterized by high blood pressure, water retention, muscle tension and insomnia (www.altmedrev.com/publications/9/2/211.pdf).

Cardiovascular Protection:

Hypoglycemic, diuretic and hypocholesterolemic effects of ashwagandha root were assessed in human subjects, in which six type 2 diabetes mellitus subjects and six mildly hypercholesterolemic subjects were treated with a powder extract for 30 days. A decrease in blood glucose comparable to that of an oral hypoglycemic drug was observed. Significant increases in urine sodium, urine volume and decreases in serum cholesterol, triglycerides and low-density lipoproteins were also seen (www.altmedrev.com/publications/9/2/211.pdf).

Hypothyroidism:

Animal studies reveal ashwagandha has a thyrotropic effect. An aqueous extract of dried Withania root was given to mice via gastric intubation at a dose of 1.4 g/kg body weight daily for 20 days. Serum was collected at the end of the 20 day period and analyzed for T3 and T4 concentrations and lipid peroxidation was measured in liver homogenate via antioxidant enzyme activity. Significant increases in serum T4 were observed, indicating the plant has a stimulatory effect at the glandular level. No changes in T3 levels were observed. Withania may also stimulate thyroid activity indirectly, via its effect on cellular antioxidant systems. Withania extract significantly decreased lipid peroxidation in the liver homogenate and significantly increased catalase activity, promoting scavenging of free radicals that can cause cellular damage. These results indicate ashwagandha may be a useful botanical in treating hypothyroidism (www.altmedrev.com/publications/9/2/211.pdf).

Other Therapeutic Considerations:

Studies show ashwagandha to be effective in the treatment of osteoarthritis, inflammation, stroke and tardive dyskinesia. Studies also reveal ashwagandha to be a potential antimicrobial agent, with antifungal activity and moderate antibacterial activity against *Staphyloccus aureus* and *Pseudomonas aeruginosa* (www.altmedrev.com/publications/9/2/211.pdf).

Dosage

A typical dose of ashwagandha is 3-6 grams daily of the dried root, 300-500 mg of an extract standardized to contain 1.5 percent withanolides or 6-12 ml of a 1:2 fluid extract per day (www.altmedrev.com/publications/9/2/211.pdf).

Warnings and Contraindications

Large doses of ashwagandha may possess abortifacient properties; therefore, it should not be taken during pregnancy. Since ashwagandha acts as a mild central nervous system depressant,

patients should avoid alcohol, sedatives and other anxiolytics while taking ashwagandha (www.altmedrev.com/publications/9/2/211.pdf).

Side effects and Toxicity

Ashwagandha is generally safe when taken in the prescribed dosage range. Large doses have been shown to cause gastrointestinal upset, diarrhea and vomiting (www.altmedrev.com/publications/9/2/211.pdf).

Withania somnifera Dunal, commonly known as ashwagandha, presents itself as a novel complementary therapy for integrative oncology care. This botanical agent has been used for centuries in Ayurvedic medicine to increase longevity and vitality (http://www.gaiaherbs.com/uploads/A_Research_Review_of_Ashwagandha-1371567339.pdf).

MATERIALS AND METHODS

Ashwagandha (*Withania somnifera*) powder (root) was purchased from ayurvedic store.

Chemicals for extraction of withanolides from Ashwagandha were methanol, n-hexane and chloroform.

Chemicals For Thin Layer Chromatography were silica powder, chloroform, methanol and iodine crystals.

Laboratory animals (Deshpande T. M. and Chaphalkar S. R. 2013)

Swiss Albino mice (*Mus musculus)* of five weeks were procured from Raj Biotech, Shirwal, Satara, Maharashtra and maintained in VSBT animal house at temperature between 25^{o}C to 35^{o}C with 12 hour light and 12 hour dark cycle and provided commercial mice feed pellets and tapwater. The study was approved by institutional animal ethics committee.

Chemicals for experiments with mice in vivo : 70% ethanol, Colchicine, Potassium Chloride, Giemsa, methanol, glacial acetic acid and Phosphate Buffered Saline (10X Stock at pH 6.8 and 1X working solution at pH 7.4), Composition for 1 liter: 8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4, 0.24g of KH2PO4 and Cyclophosphamide (LEDOXAN Dabur pharma).

Chemicals for WRL-68 cell line in vitro:

Reagents such as Dulbecco's Modified Eagle's Medium (DMEM), Trypsin Phosphate Versene Glucose (TPVG), antibiotic antimycotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin and amphotericin B 100 µg/ml), Trypan Blue, Fetal Bovine Serum (FBS) were purchased from Himedia, Mumbai, India.

Extraction of Withanolides from Ashwagandha powder:

One gram of dried powder of roots of *Withania somnifera* was extracted 2 times with 20 ml each time of the liquid extractant in an Erlenmeyer flask by shaking on a platform shaker (120 RPM) for 6 & 11 hours respectively. Solvent used for extraction was methanol:water (60:40). The extractions in the solvent composition were recovered by filtration and the filtrates from the 2 extractions in each case (of composition of extractant systems) were pooled and liquid-liquid partitioned (2 times) with (equal volume) n-hexane to remove pigments (removal of chlorophyll) (check spelling of chlorophyl and fatty materials. The defatted and depigmented extract was subjected to liquid-liquid partitioning (2 times, equal volume) to recover withanolidal fraction including withaferin A in the chloroform layer. Chloroform fractions of each extractant (solvent) systems were pooled and evaporated to dryness. The residue was dissolved in known volume (2.0 ml) of methanol/water. From 1g of root powder around 300 mg extract was obtained.

Thin Layer Chromatography (TLC) of Withanolides:

A clean grease free TLC plate was taken. The slurry of silica (50 % of silica) by dissolving the silica powder (5 grams in 10 ml of chloroform) was prepared. The slurry on TLC plate (Glass TLC plate, 60mm × 140mm) was poured and let it dry. 3 to 4 small spots of extract with capillary (inner diameter 0.8mm) at the distance of 1.5 cm from the end were given. 120 ml solvent system was added into the TLC chamber, the chamber was closed and was kept for saturation (for 30 minutes). The TLC plate was kept tilted inside the chamber till the solvent ran 10 cm from the spots applied (around 15-20 minutes). The TLC plate was removed out of the chamber when the solvent run was complete (around 15-20 minutes). The TLC plate was kept in the chamber saturated with iodine crystals. The (Retention factor) R_f values of the spots observed were calculated. The TLC plate with spots was photographed.

In vivo

Treating the mice with withanolides:

The mice were weighed and their weight was 40 gms. The dose of withanolides to be given was determined. The mice were held in left hand by holding the ear, dorsal skin and tail. The mice were inverted and the abdomen was cleaned with cotton dipped in 70% ethanol. The appropriate amount of withanolides was taken in syringe with 26G needle. The withanolides were injected intraperitoneally.

In this study, the number of mice were 5 males; comprising 3 for test group and 1 each for positive and negative control.

In Test group, three mice were injected with 3 different concentrations of withanolides; 12 mg, 28 mg, 36 mg (300, 700, 900 mg/kg).

In Positive Control, one mouse was injected with 10 mg/kg cyclophosphamide (Concentration of cyclophosphamide: 250 mg/ kg).

In Negative Control, one mouse was kept as it is without any treatment.

The mice were sacrificed after 24 hours for further studies. The number of mice for chromosomal aberration study were one for each concentration.

Dissection of mouse:

The mice was sacrificed by cervical dislocation. A cut to the abdominal skin was given. The mice were dissected from the ventral side and the testes were taken out. The hind limbs were cut and all the skin and muscles were removed from them.

Isolation of bone marrow:

The femur bone was taken out and was cut from both sides. 2 ml 0.5 %KCl was taken in to a 5 ml syringe having with 26 G needle. The needle was inserted from one end of the bone. The piston was pressed and the bone marrow was flushed out into a 15 ml falcon tube containing 3 ml 0.5 % KCl.

Chromosomal aberration study from bone marrow of mice:

Colchicine (4 mg/kg body weight) was injected intraperitoneally 2 hours before harvesting of bone marrow. The bone marrow from femur was taken out. The bone marrow was kept in 0.5% KCl for 20-30 minutes at 37^{o}C. It was centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and ice cold methanol:acetic acid (3:1) was added, mixed well and kept for 30 minutes. The small amount of this bone marrow suspension on clean grease free slides was dropped from 2 feet height. The drops were spread on slides and the slides were gently flamed dried (Deshpande et al 2008). The slides were were kept in working Giemsa stain (1 %) for 2-3 minutes. The slides were taken out and were air dried. The chromosome pairs arrested at metaphase stage were observed under 100X oil immersion objective of Zeiss phase contrast inverted microscope. Metaphase plates were photographed in digital camera with 4X zoom.

Cell line studies

The cell line WRL 68 was procured from National Centre for Cell Science (NCCS), Pune for

studying the in vitro cytotoxicity of ashwagandha root powder extract (Deshpande and Chaphalkar 2013).

As per the literature, WRL 68 (ATCC® CL48™) cell line is from human organism *Homo sapiens* with culture properties as adherent (http://www.atcc.org/products/all/CL-48.aspx#generalinformation).

Some morphologic and functional characteristics of a hepatic fetal human epithelial cell line (WRL-68 cells) were determined to validate the use of these cells as an in vitro hepatic model. WRL-68 cells have a morphologic structure similar to hepatocytes and hepatic primary cultures. They secrete alpha-feto protein and albumin and exhibit a cytokeratin pattern similar to other hepatic cultures. WRL-68 cells preserve the activity of some characteristic or specific liver enzymes or both used in clinical chemistry for the diagnosis of hepatic disorders, i.e. alanine amino transferase, aspartate amino transferase, gamma-glutamyl transpeptidase and alkaline phosphatase (Gutierrez-Ruiz MC et al 1994).

The WRL 68 (HeLa derivative) application as in vitro hepatic model, the biological source cervix from human, growth mode adherent, morphology epithelial-like. The cell line origin Human Negroid cervix carcinoma (http://www.sigmaaldrich.com/catalog/product/sigma/89121403?lang=en&region=IN).

The cell line WRL 68 mentioned as WRL 68 (HeLa derivative) (http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=89121403&collection=ecacc_gc). The human hepatic cell line WRL-68 exhibits a morphology similar to hepatocytes and hepatic primary cultures. Cells have been shown to secrete albumin and alpha-feto protein and express liver specific enzymes such as alanine amino transferase, aspartate amino transferase, gamma-glutamyl transpeptidase and alkaline phosphatase. This cell line was found to be indistinguishable from HeLa by STR PCR DNA profiling. Therefore, the cell line should be considered as derived from HeLa. Ethnicity black, species human, tissue cervix, morphology epithelial-like and growth mode adherent (http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=89121403&collection=ecacc_gc).

Microscopical observations of WRL-68 cell line morphology: After procurement of the WRL-68 cell line, initially, the color of the medium, in the T-25cm2 tissue culture flask of WRL-68 cell line was observed for an indication of pH change and whether the medium was clear without

turbidity and transparent as apparent absence of microbial contamination. The WRL-68 cell line morphology and confluency was observed and was also checked in tissue culture flask of WRL-68 cell line that there was no microbial movement and was without microbial contaminationin in Zeiss phase contrast inverted microscope at 5X, 10X and 40X magnification in phase contrast.

Daily observations of cultured WRL-68 cell line were done in Zeiss phase contrast inverted microscope at 5X, 10X and 40X magnification and were photographed with digital camera with 4X zoom.

Splitting of WRL68 cell line:

The total medium from given confluent WRL68 cell culture T-25cm2 flask was removed. 500µl of sterile TPVG was added into this flask and was rotated slowly so as to spread the sterile TPVG over the WRL-68 cells. This flask was tilted so that the free WRL-68 cells would fall down. The sterile TPVG treatment did not exceed 2 to 3 minutes. 5 ml sterile DMEM containing sterile FBS, antibiotic and antimycotic solution was added into this flask. This medium containing WRL-68 cells was taken into a sterile 50 ml falcon tube and centrifuged at 5000 rpm for 10 min. The supernatant was removed slowly without disturbing the pellet. 2 ml sterile fresh medium (sterile DMEM with sterile FBS, antibiotics and antimycotics) was added into the pellet, mixed well and 500 µl WRL-68 cell suspension from this tube was added into 4 new sterile T-25cm2 flasks each. 4.5 ml sterile fresh DMEM (with sterile FBS, antibiotics and antimycotics) was added into each flask. These flasks with WRL-68 cells were kept in CO2 incubator (at 37^{o}C and 5% CO_2) for the culture of WRL-68 cell line and attainment of the confluency. The cell count of WRL-68 cell line in T-25cm2 culture flasks was 10^5 cells/ml.

Maintenance of WRL68 cell line:

The WRL68 cell line attained the confluency after 2 days in sterile DMEM (with sterile FBS, antibiotics and antimycotics). The medium in these T-25cm2 flasks was observed for color change and the confluency of WRL-68 cell line was observed in inverted microscope at 5X, 10X and 40X magnification. If there was yellowing of the medium (yellowing of medium occurs when there is overgrowth of the cells) or confluency was attained then subcultured the WRL-68 cell line in these flasks by the following method. The total medium from these T-25cm2 flasks containing cultured confluent WRL68 cell line was removed. 500 µl sterile TPVG was added into these flasks and were rotated slowly so as to spread the sterile TPVG over the WRL-68 cells. These flasks were tilted so that the free WRL-68 cells would fall down. The sterile TPVG

treatment did not exceed 2 to 3 minutes. 5 ml sterile DMEM containing sterile FBS, antibiotics and antimycotics was added into these flasks. This medium containing WRL-68 cells was taken into a sterile 50 ml falcon tube and centrifuged at 5000 rpm for 10 min. The supernatant was removed slowly without disturbing the pellet. 2 ml sterile fresh medium (sterile DMEM with sterile FBS, antibiotics and antimycotics) was added into the pellet, mixed well and 500µl WRL-68 cell suspension from this tube was added into 4 new sterile T-25cm2 flasks each. 4.5 ml sterile fresh DMEM (with sterile FBS, antibiotics and antimycotics) was added into each of these flasks and these flasks with WRL-68 cells were kept in CO2 incubator at 370C and 5% CO2 for the culture of WRL-68 cell line and attainment of the confluency. The cell count of WRL-68 cell line in T-25cm2 culture flasks was 10^5 cells/ml.

Culturing of cells in sterile 24 well plate:

A T-25cm2 flask containing cultured confluent WRL-68 cell line was taken. The total medium from this T-25cm2 flask containing confluent WRL68 cell line was removed. 500µl sterile TPVG was added into this flask and was rotated slowly so as to spread the sterile TPVG over the WRL-68 cells. This flask was tilted so that the free WRL-68 cells would fall down. The sterile TPVG treatment did not exceed 2 to 3 minutes. 5 ml sterile DMEM containing sterile FBS, antibiotics and antimycotics was added into this flask. This medium containing WRL-68 cells was taken into a sterile 50 ml falcon tube and centrifuged at 5000 rpm for 10 min. The supernatant was removed slowly without disturbing the pellet. 2 ml sterile fresh medium (sterile DMEM with sterile FBS, antibiotics and antimycotics) was added into the pellet, mixed well and 50 µl cell suspension from this tube was added into the wells of sterile 24 well plate. 450 µl sterile fresh DMEM (with sterile FBS, antibiotics and antimycotics) was added into each well of this sterile 24 well plate. Then, this 24 well plate with WRL-68 cells and sterile complete medium was kept in CO2 incubator at 370C and 5% CO2 for the culture of WRL-68 cell line and attainment of the confluency. The cell count of WRL 68 cell line in sterile 24 well plate was $5X10^4$ cells / ml.

Treating the WRL-68 cell line in sterile 24 well plate with withanolides:

The WRL-68 cell line cultured in the wells of sterile 24 well plate was treated with 4 different concentrations of withanolides; 2 mg, 6 mg, 10 mg and 20 mg per well. These broad range of concentrations were chosen that can be tolerated by cultured cells. The concentration of the extract was around 165µg/µl so the volume accordingly for 2 mg, 6 mg, 10 mg and 20 mg was

used. The sterile 24 well plate containing WRL-68 cell line cultured for 24 hours was taken and 12 µl, 36 µl, 60 µl and 120 µl of the extract (dissolved in water) was added into 16 wells (4 wells each). 20 µl cyclophosphamide was added into 4 wells (positive control) and 4 wells were kept as it is (negative control). This sterile 24 well plate containing WRL-68 cell line treated with withanolides and cyclophosphamide was kept in CO2 incubator at 370C and 5% CO2. The WRL-68 cell line in this 24 well plate was observed after 24 hours for change in morphology and viability.

Trypan blue dye exclusion staining of WRL68 cell line:

500 µl 0.4% trypan blue stain was added in each well of the 24 well plate and let it stand for 5 minutes. The color of the WRL-68 cells was observed. Dead cells got stained with dark blue color and viable cells remained unstained.

OBSERVATIONS

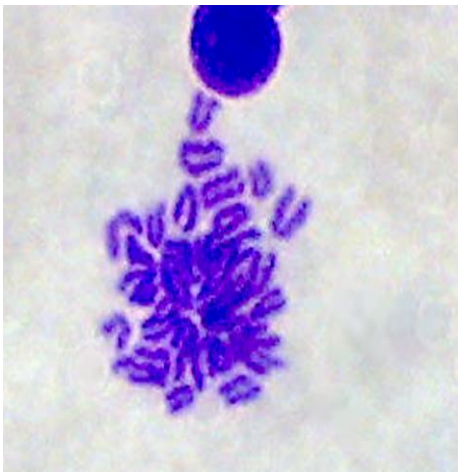
TLC:

After development of plate in iodine chamber 7 yellowish brown spots were observed.

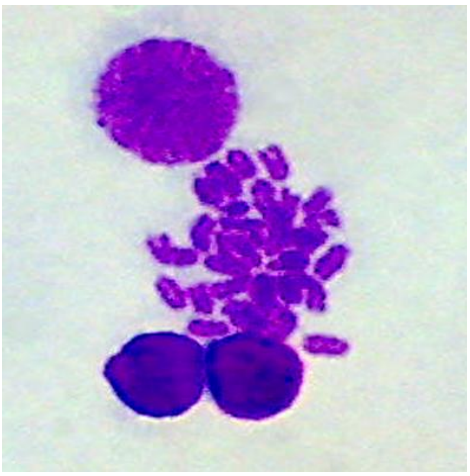
The Rf values were: 0.04, 0.3, 0.6, 0.73, 1.24, 0.9

IN VIVO STUDIES :

MICE CHROMOSOME ABERRATION TEST



Chromosomes of Normal mouse (negative control)

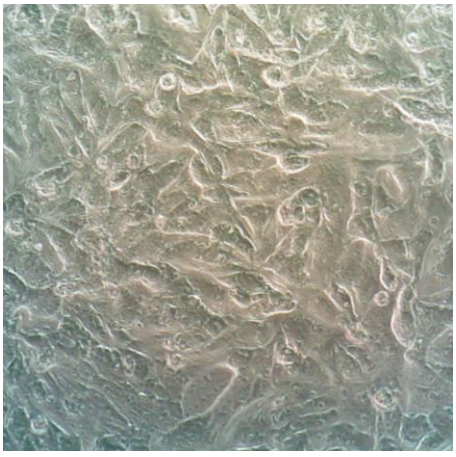


Chromosomes of Withanolides treated mouse (test) with 36mg Ashwagandha

In negative control, normal V shaped chromosomes were observed and in mice treated with withanolides, no significant chromosomal damages were observed.

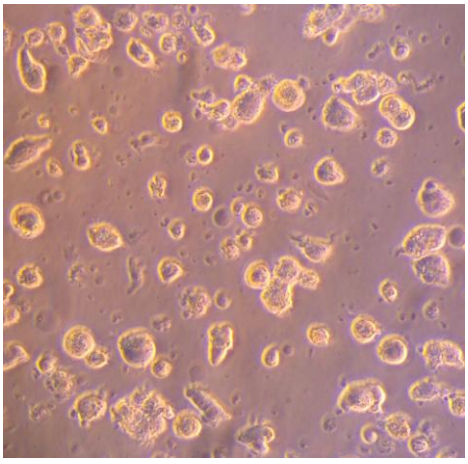
WRL-68 CELL LINE STUDY :

Initial confluent WRL68 cell line



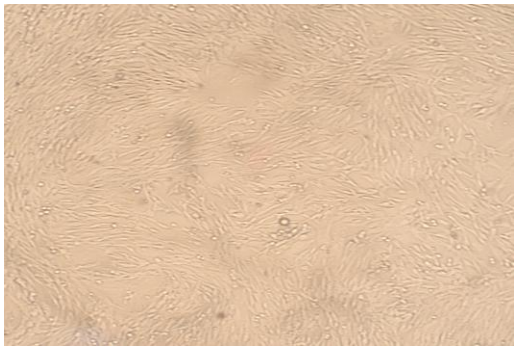
40 X

WRL68 cells immediately after subculture



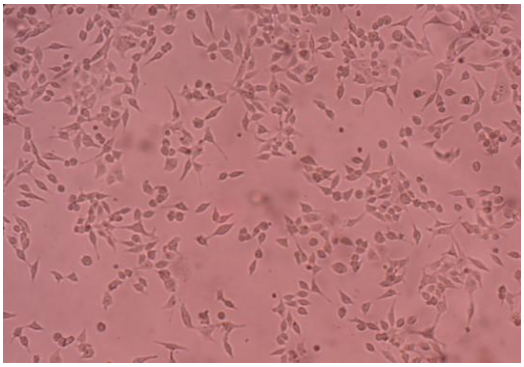
40X

Confluent WRL68 cells in DMEM medium (with serum & antibiotics) after 2 days incubation



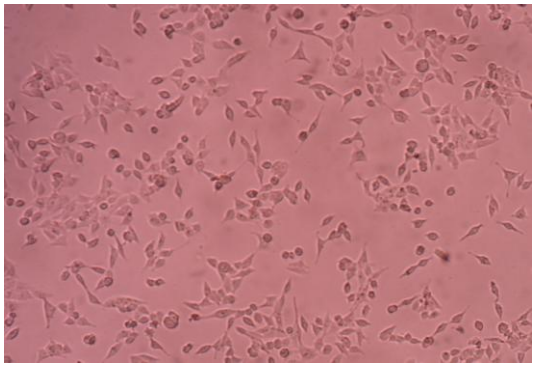
10X

Normal WRL68 cells



10X

The WRL-68 cell line at time zero

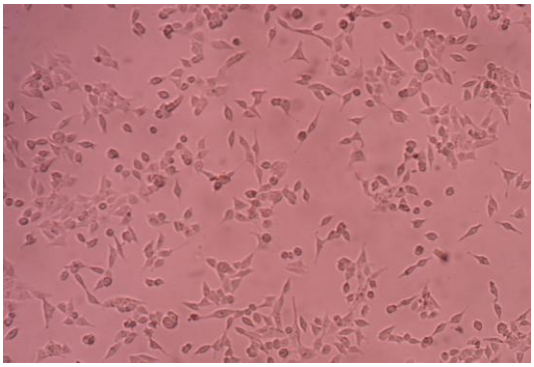


10X

The WRL-68 cell line at 24 hours

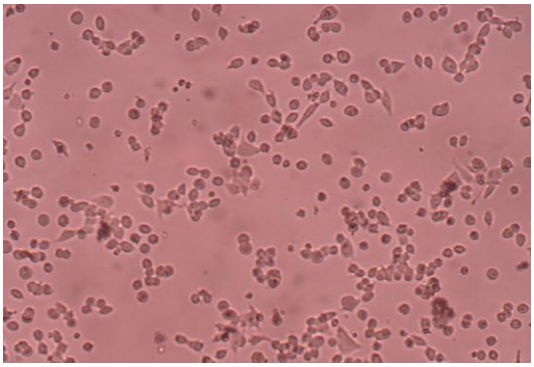
The WRL-68 cell line before treatment was confluent, adherent, healthy with epithelial like morphology, with many bipolar and tripolar cells in clusters, someplaces in flask with cytoplasmic process of 1 cell joined to the other cell like dumbbell shaped (2 cells and 3 cells joined together) and some round cells.

WRL68 cells treated with cyclophosphamide



10X

Before treatment

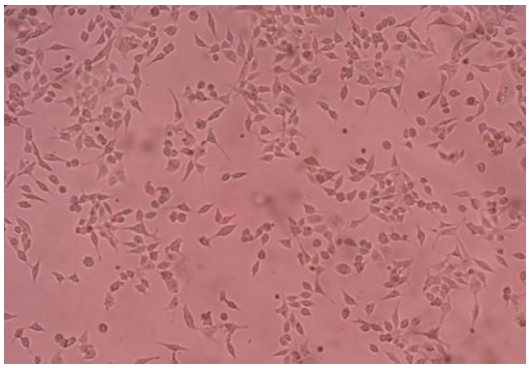


10X

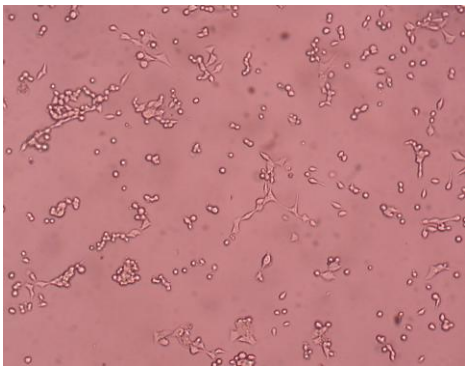
After treatment1µM final concentration

WRL-68 cell line treated with cyclophosphamide was not confluent, cells viable, detached from substratum, rounded, in clumps, not like epithelial and many cells without cytoplasmic processes as compared to before treatment at time zero.

WRL68 cells treated with Ashwagandha



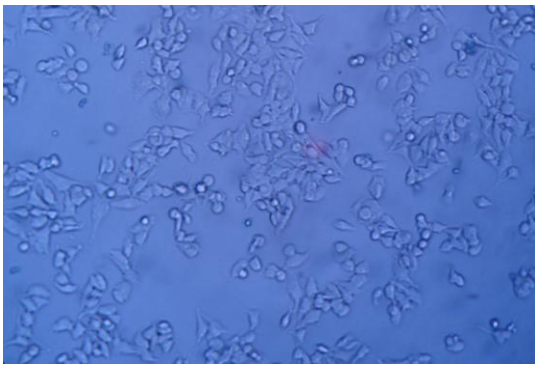
10X
Before treatment



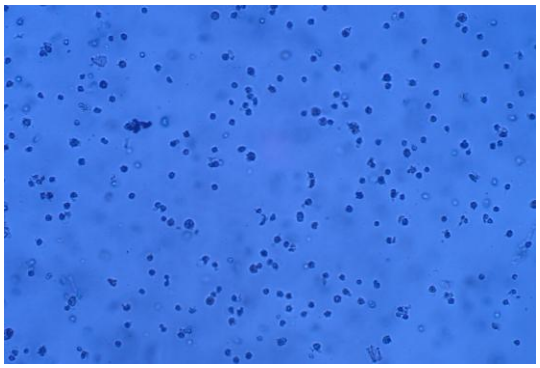
10X
After treatment with 36µl
of withanolides extract

WRL-68 cell line treated with ashwagandha caused the detachment of the cells from the bottom surface. The Trypan blue staining showed that all Ashwagandha treated cells were dead.

Trypan blue viability staining of WRL-68 cell line



10X
Viable cells (unstained)



10X
Dead cells (stained)

CONCLUSION

As the in-vivo study showed that there is no significant chromosomal damage and not many sperm shape abnormalities due to Ashwagandha in mice, it can be concluded from this preliminary study that the Ashwagandha root powder extract is non genotoxic

In the cell line study, the Ashwagandha extract caused the death of cells but this may be due to the high concentrations of extract and direct action of components of the extract onto the cultured cells, in contrast, in the in-vivo system, there is natural detoxification and dilution of the extract inside the body of mice.

DISCUSSION

In a similar study of Guruprasad et al 2010, to assess the role of Brahma rasayana (BR) (which included ashwagandha among other plants) on genotoxicity in vivo in a mouse test system, there was no genotoxicity reported due to ashwagandha in vivo in mice. Furthermore, sperm abnormalities and chromosomal aberrations were insignificant in the treatment group when compared to controls. However, there was a marginal increase in sperm count in the BR treated animals. These findings clearly indicate that there are no observed adverse genotoxic effects elicited by BR in experimental animals such as mice (Guruprasad et al 2010). BR does not elicit genotoxic effects in the mouse system, rather that it is a moderate enhancer of reproductive and mitotic cellularity, and a protector against certain kinds of sperm abnormality (Guruprasad et al 2010). Guruprasad et al 2010 reported the non genotoxic effect of mixture of plant extracts (BR) which included ashwagandha extract. Our results of preliminary in vivo studies of ashwagandha root extract in mice on chromosome morphology too are in agreement with (Guruprasad et al 2010) that ashwagandha is non genotoxic in vivo in mice.

As per Bernhardt et al (2011), ashwagandha can be effective in preventing the genotoxic effects of commercial grade malathion (CGM) in vivo in mice. Dismutagenics are capable of preventing the action of damage inducing agents, mainly by adsorption of them. The bioantimutagenic agents act within the cell and are instrumental in injury prevention or the DNA repairs (Kada and Shimoi 1987; Oliveira et al 2006). WS acts both by dismutagenic and bioantimutagenic mechanism, its activity as an antioxidant strengthens its dismutagenic action. WS possibly has the therapeutic power to prevent the genotoxic damage caused by CGM by its action as a free radical scavenger (Bernhardt et al 2011).

Thus, *Withania somnifera* is non genotoxic as per our preliminary observations and as per Bernhardt et al (2011) is also antigenotoxic.

Withaferin A, also showed significant antitumor & radiosensitizing effects in experimental tumors without any toxicity & inhibiting tumor growth increasing survival in swiss albino mice inoculated with Ehrlich ascites (ESC) carcinoma (Sharma et al 2011).

Suppressive effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice was reported (Davis and Kuttan 1998). Administration of *Withania somnifera* extract (Solanaceae) was found to significantly reduce leucopenia induced by cyclophosphamide (CTX) treatment. Treatment of Withania along with CTX was found to significantly ($P < 0.001$)

increase the bone marrow cellularity (13.1 x 10(6) cells/femur) compared to CTX alone treated group (8 x 10(6) cells/femur).The major activity of *Withania somnifera* may be the stimulation of stem cell proliferation. *Withania somnifera* could reduce the cyclophosphamide induced toxicity and its usefulness in cancer therapy (Davis and Kuttan 1998)

The Withaferin A (WA) treatment decreased viability of MCF-7 (estrogen-responsive) and MDA-MB-231 (estrogen-independent) human breast cancer cells in a concentration-dependent manner. The WA-mediated suppression of breast cancer cell viability correlated with apoptosis induction characterized by DNA condensation, cytoplasmic histone-associated DNA fragmentation and cleavage of poly-(ADP-ribose)-polymerase. On the other hand, a spontaneously immortalized normal mammary epithelial cell line (MCF-10A) was relatively more resistant to WA-induced apoptosis compared with breast cancer cells. The MCF-10A cell line was isolated from fibrocystic breast disease and was spontaneously immortalized. The MCF-10A cells have intact cell cycle checkpoints and normal proliferation controls. The human breast cancer cells were relatively more sensitive to apoptosis induction by WA compared with a normal mammary epithelial cell line and a spontaneously immortalized and nontumorigenic normal mammary epithelial cell line (MCF-10A) is relatively more resistant to WA-induced apoptosis compared with MCF-7 and MDA-MB-231 cells (Stan et al 2008). Thus, as per Stan et al (2008), ashwagandha may be non cytotoxic to normal mammary epithelial cells line (MCF-10A) compared to MCF-7 and MB-231 human breast cancer cell lines as the normal mammary epithelial cells are resistant to WA induced apoptosis than breast cancer cell lines.

Toxicity studies reveal that ashwagandha appears to be a safe compound. Preliminary studies have found various constituents of ashwagandha exhibit a variety of therapeutic effects with little or no associated toxicity. These results are very encouraging and indicate this herb should be studied more extensively to confirm these results and reveal other potential therapeutic effects. (http://www.altmedrev.com/publications/5/4/334.pdf)

In one long-term study, WS was boiled in water and administered to rats in their daily drinking water for eight months while monitoring body weight, general toxicity, well being, number of pregnancies, litter size, and progeny weight. The estimated dose given was 100 mg/kg/day. In the second part of the study, the estimated dose was 200 mg/kg/day given for four weeks as above while monitoring body temperature, body weight, cortisol value in heparinized plasma and ascorbic acid content of the adrenals. The liver, spleen, lungs, kidneys, thymus, adrenals and

stomach were examined histopathologically and were all found to be normal. The percent weight gain after eight weeks on the same WS treatment was 227 percent for the treated group and 145.3 percent in the control group. The relative body weight gain was significantly greater in the WS-treated group as compared to the control group ($p < 0.001$). (http://www.altmedrev.com/publications/5/4/334.pdf)

In the four-week study, the weight gain in the treated group was comparable to that of the control group. The body temperature in the WS treated group was 1.7ºC lower than the controls. The WS treatment caused an increase in lung and liver weights and a decrease in adrenocortical activity as was evident from the reduction in adrenal weight and a significant reduction in plasma cortisol ($p<0.001$). Histopathologically, all organs were normal. The authors attributed the increase in liver weight to an increase in glycogen storage because WS contains many steroids and glucocorticoids known to enhance liver glycogen stores. Reduction in metabolic rate also leads to underutilization of glycogen stores in the liver, leading to its accumulation. The reduced adrenocortical activity may be attributed to steroid moieties in WS roots which may act like exogenous adrenocortical steroids, lowering the ACTH (AdrenoCorticoTropicHormone) secretion and consequently, endogenous steroid production. The authors concluded the decoction of WS promoted growth especially during the active growth period and helped produce healthier progeny. The WS group was devoid of any toxic effects after eight months of daily dosing in this study. (http://www.altmedrev.com/publications/5/4/334.pdf)

Primary hepatocytes were immortalised with Moloney's mouse leukaemia virus expressing E6 and E7 proteins of human papillomavirus, and cultures propagated long-term. These (human hepatocyte lines) HHLs retain primary hepatocyte phenotype and should be useful for investigating mechanisms of entry and replication of hepatotropic viruses, and should also be valuable in the study of hepatocyte biology and pathology (Clayton et al 2005). Thus, lower concentrations of ashwagandha extract than those from this preliminary study may be done to check for toxicity on WRL-68 cell line and on other normal human liver cell lines.

ACKNOWLEDGEMENTS

The authors are thankful to Mr. Prasad B. Abnave M.Sc. Biotechnology student of 2006-2008 batch for part contribution.

LITERATURE CITED

1. www.altmedrev.com/publications/9/2/211.pdf
2. http://www.altmedrev.com/publications/5/4/334.pdf
3. http://www.gaiaherbs.com/uploads/A_Research_Review_of_Ashwagandha-1371567339.pdf
4. Deshpande T. M. and Chaphalkar S. R.2013 A Preliminary Twenty Eight Days Repeated Dose Cytotoxicity Test of Polyherbal Formulation as ANTI-HIV Herbal Drug International Journal of Advances in Pharmacy, Biology, Chemistry 2(3), ISSN: 2277 – 4688.
5. Deshpande T. M., Shyama S. K. and Pandey A. K. 2008 Genetic Studies of Breast Cancer Patients in Goa, India Int J Hum Genet, 8(3): 263-268.
6. Deshpande T.M. and Chaphalkar S. R. 2013 Antiviral activity of plant extracts against FMDV in vitro a preliminary report International Journal of Institutional Pharmacy and Life Sciences 3(4): July-August, (ISSN): 2249-6807.
7. http://www.atcc.org/products/all/CL-48.aspx#generalinformation
8. Gutiérrez-Ruiz MC, Bucio L, Souza V, Gómez JJ, Campos C, Cárabez A. 1994 Expression of some hepatocyte-like functional properties of WRL-68 cells in culture. In Vitro Cell Dev Biol Anim. Jun;30A(6):366-71.
9. http://www.sigmaaldrich.com/catalog/product/sigma/89121403?lang=en&region=IN
10. http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp
11. Guruprasad K.P., Mascarenhas R., Gopinath P.M., and Satyamoorthy K. 2010 Studies on Brahma rasayana in male swiss albino mice: Chromosomal aberrations and sperm abnormalities J Ayurveda Integr Med. Jan-Mar; 1(1): 40–44.
12. Bernhardt V., D'Souza J., Shantaram M. 2011 In vivo genetic damage induced by commercial Malathion and the antigenotoxic role of *Withania somnifera*. International Journal of Integrative Biology, 11 (2), 78-84.
13. Sharma V., Sharma S., Pracheta, Paliwal R. 2011 Withania somnifera: A Rejuvenating Ayurvedic Medicinal Herb for the Treatment of various Human ailments International Journal of PharmTech Research CODEN (USA) 3(1): IJPRIF 3(1) Jan-Mar, 187-192, ISSN : 0974-4304.
14. Davis L, Kuttan G. 1998 Suppressive effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. J Ethnopharmacol. Oct;62(3):209-14.
15. Stan S.D., Hahm E.-R., Warin R., and Singh S.V. 2008 Withaferin A Causes FOXO3a- and Bim-Dependent Apoptosis and Inhibits Growth of Human Breast Cancer Cells In Vivo. Cancer Res. Sep 15; 68(18): 7661–7669.
16. Clayton R.F., Rinaldi A., Kandyba E.E., Edward M., Willberg C., Klenerman P., Patel A.H. 2005 Liver cell lines for the study of hepatocyte functions and immunological response. Liver Int. 2005 Apr;25(2):389-402.