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Validation Of Withaferin A By High Performance Liquid Chromatography And Study Of Cytotoxicity By MTT Assay On Breast Cancer Cell Line

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Abstract
Background: *In the present time, attention of people has been changed from manmade (synthetic) to natural medicines. Many quality control tools are there which are used to confirm the excellence of herbal drugs. HPLC (High performance liquid chromatography) is one of them. So for this reason validation of the compound is very much essential step for the quality guarantee. Notably, breast cancer alone contributes to 30% of female cancer cases. MCF-7 breast cancer cells are adherent cells, which means they need to attach to a surface for the invitro growth. MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a water-soluble, yellow dye that is used to measure cell viability and cytotoxicity. In this present study both breast cancer cells (MDA-MB-231 and MCF-7) are treated with Withaferin A to know the apoptotic effect by MTT dye.*
Objective: *To validation the Withaferin A by high performance liquid chromatography and study of cytotoxicity by MTT assay on breast cancer cell line.*
Materials and Methods: *Withaferin A was purchased from ELECTROCRAFTS (FBD), Faridabad. All chemicals and solvents were used HPLC grade and obtained from E-Merck and other renowned companies. Other materials required are Breast cancer cell lines, DMEM complete media, 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10µg/mL insulin. Breast cancer cells were treated with different concentration of Withaferin A. Cytotoxicity was measured by MTT assay. The morphological change of untreated (Control) and treated cells were observed under digital inverted microscope and photographed.*
Result: *Sample and standard shows a good resolution, sharp and symmetrical peak at retention time 13.685 min. and 13.600 min. respectively. At the lower concentration less cytotoxicity and at higher concentration more cytotoxicity of Withaferin A occurs in breast cancer cell.*
Conclusions: *Withaferin A has anticancer potential.*
Key Words: *Breast cancer cell line, Withaferin A, HPLC, cytotoxicity.*

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I. Introduction

In the present time, attention of people has been changed from manmade (synthetic) to natural medicines. With increasing the need of natural products it is essential to keep the quality of them for benefit of human being [1]. Many quality control tools are there which are used to confirm the excellence of herbal drugs. HPLC (High performance liquid chromatography) is one of them [2]. So for this reason validation of the compound is very much essential step for the quality guarantee.

Cancer counts modelled at various levels, including national and state, were combined yearly, and a time series projection technique (vector auto regression) was utilized across the 15-year span to forecast cancer cases for the year 2020. Projected cancer-related fatalities in 2020 were determined by estimating the annual percent change in reported cancer deaths from 2003 to 2017, utilizing join point analysis at state and national levels, as reported to the National Center for Health Statistics (NCHS). For a comprehensive understanding of this methodology, please consult [3]. Among women, the three most prevalent cancers are breast, lung, and colorectal, collectively representing half of all new diagnoses. Notably, breast cancer alone contributes to 30% of female cancer cases [4]. The gender gap in cancer incidence exhibits age-dependent variations. In childhood (ages birth -14 years), incidence is about 10% higher in males compared to females (18.2 vs. 16.4 per 100,000 population). Conversely, during early adulthood (ages 20-49 years), the incidence is notably 77% higher in females (203.4 vs. 114.9 per 100,000 population), primarily attributable to the incidence of breast cancer in young women [4]. The marginal increase in breast cancer incidence rates (around 0.3% per year) since 2004 is linked, in part, to ongoing declines in fertility rates and rising obesity. These factors are also associated with the sustained rise in incidence

for uterine corpus cancer, showing a yearly increase of 1.3% from 2007 to 2016 [4]. Breast cancer stands as a significant global health concern, impacting the lives of numerous women across the world. In 2020, over 40,000 women in the United States were anticipated to succumb to the challenges posed by breast cancer [5]. There remains a crucial need for innovative therapeutic and preventive approaches to reduce both mortality and the burden of suffering associated with this disease. Ongoing research explores extract from medicinal plants or their small- molecule constituents as potential novel strategies for the therapy and/or chemoprevention of breast cancer.

Ashwagandha is the common name of *Withania somnifera* most precious herbaceous plant in the conventional systems of Indian medicine having many valuable effects [6]. This one is also called "Indian Winter cherry" or "Indian Ginseng" [7]. It is one of desert plant which grew up in desiccated and rain-forest area [8]. One of the active chemical component of *Withania somnifera* is Withaferin A [9]. *Withania somnifera* belonging to the solanaceae family, is a compelling medicinal plant currently undergoing rigorous investigation for its potential impact on cancer and various other health conditions. The root/leaf extract of *Withania somnifera* remains a key component in the formulation of Ayurveda, Siddha and Unani medicine practices, prevalent in India and neighbouring countries [10][11][12][13][14]. In addition to being used as a treatment for insomnia, anxiety, convulsions, skin disorders, inflammatory conditions, nervous exhaustion, impotence, improving memory or cognitive function, and increasing insulin secretion, it has been demonstrated to be a helpful treatment for cancer cells [15].

Demand of Ayurvedic origination is raise more in current years in world market because the bioactive component of *withania somnifera* (Withaferin A) are nontoxic, safe and cost effective with less side effects [16]. While the anticancer potential of each identified chemical component in *Withania somnifera* extract is still under investigation, Withaferin A, member of the withanolide family, has been the subject of extensive research for its anti- cancer effects across various types, notably in breast cancer [17][18][19][20][21][22][23].

According to demand and popularity of Ayurvedic preparation it is important to develop validated bioactive component of *withania somnifera* for safety, efficacy and quality. Validation step of quality control provides the more security and increase the faith of customer on Ayurvedic preparation. It is quite tedious work to launch the quality control parameters for validation due to the intricate type and in-built variability of the bioactive compound of *withania somnifera* (Withaferin A).

ClinicalTrials.gov lists over 15 ongoing clinical trials utilising *Withania somnifera* extract for diverse medical conditions [5]. Studies have explored the clinical effects of *Withania somnifera* extract, examining its potential in managing male reproductive functions, neuroprotection, alleviation of stress and anxiety, enhancement of memory and cognitive functions, muscle strength, and recovery, among other areas [12] [14] [25] [26]. *Withania somnifera* extract is accessible without a prescription in the United States, offered as a dietary supplement over the counter. The phytochemical composition of Withania somnifera extract is notably diverse, evident in the presence of withanolides, alkaloids, and sitoindosides [28].

II. Material And Methods

High Performance Liquid Chromatography

Plant sample

The dried ashwagandha (W. somnifera) plant sample (root) was collected from Harberium (Government Ayurvedic college and hospital), Patna, Bihar. Dried ashwagandha sample was minced and grinded with a mechanical grinder (Hanil Co. Seoul, South Korea) into a mesh size 120 mm and stored at 4°C, until further analysis.

Reagents and standards

The standard Withaferin A was purchased from ELECTROCRAFTS (FBD), Haryana, India. All of the chemicals and solvents were purchased from E-Merck and other reputable vendors and were either analytical quality or HPLC grade.

HPLC Analysis

Instrumentation

HPLC instrumentation & chromatographic conditions

HPLC analysis was performed on Shimadzu modal no. CBM-20A lite with Shimadzu's Lab Solutions software. Shimadzu High Performance Liquid Chromatographic system equipped with quaternary pump, Auto sampler, oven, detector. Separation was achieved on RP-C18, model LC-20AD of length 150mm columan. The mobile phase was methanol and used in an isocratic mode with flow rate of 1.8ml/min, 10µl of the test sample (triplicate)was injected to HPLC system.

Autosampler - Sample Rack 1.5 mL 105 vials, Control Vial Needle Stroke 52 mm, Rinsing Volume 500 uL, Needle Stroke 52 mm, Rinsing Speed: 35 uL/sec, Model SIL 20AC, Sampling Speed: 15 uL/sec, Purge Time: 25.0 min, Rinse Dip Time: 3 sec, and Cooler Temperature: 15°C were utilized. The oven, model CTO-10ASvp,

had a maximum temperature of 85°C and an oven temperature of 32°C. PDA Model SPD-M20A, Lamp D2, Cell Temperature 40°C, Start Wavelength 190 nm, and End Wavelength 800 nm were utilized.

Standard preparation – Dissolve 1mg standard compound (Withaferin A) in 1ml methanol and make 50ppm.

Sample preparation – Dissolve 19.7mg in 10 ml methanol from this solution 0.1ml was taken and dissolve in 0.9ml methanol.

The following optimized chromatographic settings were used to achieve good separations and an appropriate retention duration of Withaferin A in isocratic elution:
Column: C18 ,150mm.
Detection: 227 nm wavelength.
Detector: Photodiode Array (PDA).
Mobile phase: Methanol
Flow rate: 1.8 ml/min.
Injection volume: 10 µl.
Mode of Operation: Isocratic elution.
Run time: 30 min

MTT ASSAY ((3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide)
Reagents

MCF-7 cells were obtained from NCCS, Pune. Gentamicin Gibco, Life Technologies, MEM nonessential amino acids, and 10% fetal bovine serum (FBS) are added to DMEM complete medium. 10µg/mL insulin obtained from Sigma-Aldrich. 0.25% (w/v) Trypsin0.53 mM EDTA solution were obtained from GeminiBio. MTT were obtained from Himedia. Withaferin A was obtained from Cayman chemicals.

Cell line culture

MCF-7 breast cancer cell lines were grown adherently and maintained in DMEM complete media supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10µg/mL insulin in a 37°C incubator with 5% CO2 and 95% air. Media was changed every 2–3 days and cells were passaged at 65–80% reached confluency. 2.4.3. Trypsinization Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Add 1.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 minutes). Cells that are difficult to detach can be placed at 37°C to facilitate dispersal. Add 2.0-3.0 mL of complete growth medium and aspirate cells by gently pipetting. Transfer the cell suspension to the centrifuge tube with the medium and centrifuge at 1000 rpm for 5-10 minutes. Discard the supernatant. In the new growth media, resuspend the cell pellet. Add appropriate aliquots of the cell suspension to well plates.

Trypsinization

To get rid of any residues of serum containing trypsin inhibitor, quickly rinse the cell layer with a 0.25% (w/v) Trypsin-0.53 mM EDTA solution. Add 1.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 minutes). Cells that are difficult to detach can be placed at 37°C to facilitate dispersal. Add 2.0-3.0 mL of complete growth medium and aspirate cells by gently pipetting. Transfer the cell suspension to the centrifuge tube with the medium and centrifuge at 1000 rpm for 5-10 minutes. Discard the supernatant. Resuspending the cell pellet in the fresh growth media is necessary. Add appropriate aliquots of the cell suspension to well plates.

Method

The cells were seeded in 96-well microplates (1x106 cells/well) and incubated at 37°C for 48 hrs. in 5% CO_2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the calls were treated with different concentration of samples and incubated for 24hrs. The morphological changes of untreated (Control) and the treated cell were observed under digital inverted microscope (40X magnification) after 24 hrs. and photographed. The cell was then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 ul of (MTT) solution (5mg/mL in PBS) was added to each well. The plates were then stand at 37°C in dark for 2 hrs. The formazan crystals were dissolved in 100ul DMSO and the absorbance was read spectrophotometrically at 570 nm.

Cytotoxicity assays

The cytotoxicity effect of was tested against MCF-7 cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

III. Result And Discussion

HPLC

The sample and standard solutions were run in methanol mobile phase systems. It was found that after running the sample and standard a good resolution, sharp and symmetrical peak at retention time 13.685 min. (Figure no.1) and 13.600 min.(Figure no.2) respectively was obtained.

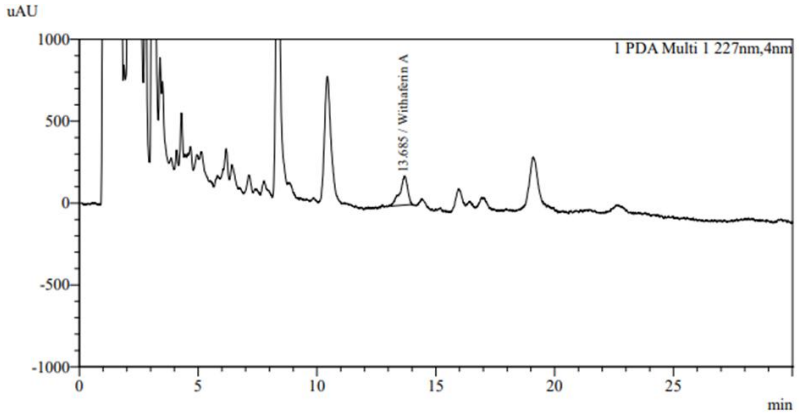

Figure no 1: Retention time of sample

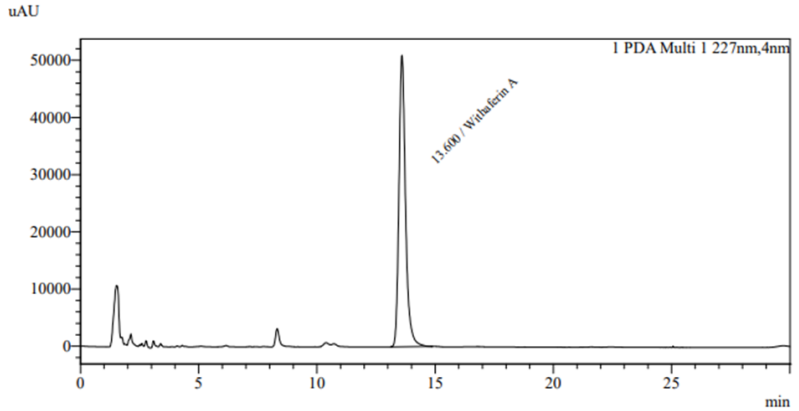

Figure no 2: Retention time of standard

MTT Assay (Cytotoxicity)

Methanolic crude extract of root powder of *Withania somnifera* treated on the different concentration of breast cancer cells. Different concentration of sample 31.25, 62.5, 125, 250, 500 ug/ml treated with MCF-7 cell lines. It was found that at low concentration of sample the percentage viability on breast cancer cell lines was high and inhibition percentage was low. At highest concentration of sample, the percentage viability on breast cancer cell lines was low and percentage of inhibition was high. In control cell viability was 100% and inhibition was 0% in both MCF-7 breast cancer cell line. At concentration of 31.25, 62.5, 125, 250, 500 ug/ml of sample the percentage cell viability was 91.766, 75.830, 40.637, 25.232, 11.886 on MCF-7 cell lines. At concentration of 31.25, 62.5, 125, 250, 500 ug/ml of sample the percentage inhibition was 8.234, 24.170, 59.363, 74.768, 88.114 on MCF-7 cell lines.

Table no 1: O.D value of MCF-7 cell line

Concentrations (µg/mL)	Absorbance		Average	Cell Viability (%)	Inhibition %
	I	II			
Control	0.745	0.761	0.753	100	0
31.25	0.685	0.697	0.691	91.766	8.234
62.5	0.58	0.561	0.571	75.830	24.170
125	0.31	0.301	0.306	40.637	59.363
250	0.183	0.196	0.190	25.232	74.768
500	0.097	0.082	0.090	11.886	88.114
Withaferina 500 µg/ml	0.074	0.069	0.072	9.562	90.438

References

[1]. Sharma, V., Gupta, A. P., Bhandari, P., Gupta, R. C., & Singh, B. (2007). A Validated And Densitometric HPTLC Method For The Quantification Of Withaferin-A And Withanolide-A In Different Plant Parts Of Two Morphotypes Of Withania Somnifera. Chromatographia, 66(9–10), 801–804. Https://Doi.Org/10.1365/S10337-007-0396-2

[2]. Balekundri, A., & Mannur, V. (2020). Quality Control Of The Traditional Herbs And Herbal Products: A Review. Future Journal Of Pharmaceutical Sciences, 6(1). Https://Doi.Org/10.1186/S43094-020-00091-5

[3]. L. Wan, Ruiqian, Weigand, "基因的改变NIH Public Access," J Neurochem, Vol. 4, No. 4, Pp. 1–15, 2015, Doi: 10.1002/Cncr.27404.Predicting.

[4]. R. L. Siegel And K. D. Miller, "Cancer Statistics, 2020," Vol. 70, No. 1, Pp. 7–30, 2020, Doi: 10.3322/Caac.21590.

[5]. E. Hahm, S. Kim, K. B. Singh, K. Singh, And V. Shivendra, "HHS Public Access," Vol. 13, No. 9, Pp. 721–734, 2021, Doi: 10.1158/1940-6207.CAPR-20-0259.A.

[6]. Gupta, G., & Rana, A. (2007). PHCOG MAG.: Plant Review Withania Somnifera (Ashwagandha): A Review Girdhari. Withania Somnifera, 1(1), 129–136.

[7]. Singh, N., Bhalla, M., De Jager, P., & Gilca, M. (2011). An Overview On Ashwagandha: A Rasayana (Rejuvenator) Of Ayurveda. African Journal Of Traditional, Complementary And Alternative Medicines, 8(5 SUPPL.), 208–213. Https://Doi.Org/10.4314/Ajtcam.V8i5s.9

[8]. Meher, S. K., Das, B., Panda, P., Bhuyan, G. C., & Rao, M. M. (2016). Uses Of Withania Somnifera (Linn) Dunal (Ashwagandha) In Ayurveda And Its Pharmacological Evidences. Research Journal Of Pharmacology And Pharmacodynamics, 8(1), 23. Https://Doi.Org/10.5958/2321-5836.2016.00006.9

[9]. Saleem, S., Muhammad, G., Hussain, M. A., Altaf, M., & Abbas Bukhari, S. N. (2020). Withania Somnifera L.: Insights Into The Phytochemical Profile, Therapeutic Potential, Clinical Trials, And Future Prospective. Iranian Journal Of Basic Medical Sciences, 23(12), 1501–1526. Https://Doi.Org/10.22038/Ijbms.2020.44254.10378

[10]. D. L. Palliyaguru, S. V Singh, And T. W. Kensler, "HHS Public Access," Vol. 60, No. 6, Pp. 1342–1353, 2017, Doi: 10.1002/Mnfr.201500756.Withania.

[11]. R. Nasimi Doost Azgomi Et Al., "Effects Of Withania Somnifera On Reproductive System: A Systematic Review Of The Available Evidence," Biomed Res. Int., Vol. 2018, 2018, Doi: 10.1155/2018/4076430.

[12]. J. Sarris, "Herbal Medicines In The Treatment Of Psychiatric Disorders: 10-Year Updated Review," Phyther. Res., Vol. 32, No. 7, Pp. 1147–1162, 2018, Doi: 10.1002/Ptr.6055.

[13]. S. Srivastav, M. Fatima, And A. C. Mondal, "Important Medicinal Herbs In Parkinson's Disease Pharmacotherapy," Biomed. Pharmacother., Vol. 92, Pp. 856–863, 2017, Doi: 10.1016/J.Biopha.2017.05.137.

[14]. K. Chandrasekhar, J. Kapoor, And S. Anishetty, "A Prospective, Randomized Double-Blind, Placebo-Controlled Study Of Safety And Efficacy Of A High-Concentration Full-Spectrum Extract Of Ashwagandha Root In Reducing Stress And Anxiety In Adults," Indian J. Psychol. Med., Vol. 34, No. 3, Pp. 255–262, 2012, Doi: 10.4103/0253-7176.106022.

[15]. Joshi, K. (2016). Studies Of Ashwagandha (Withania Somnifera Dunal) Anti-Fatigue Formula View Project SAMRAS Project View Project. International Journal Of Pharmaceutical & Biological Archives, 7(January), 1–11. Https://Www.Researchgate.Net/Publication/303343480

[16]. Arun Rasheed, K.V. Satyanarayana, P. S. G. And M. S. R. (2013). Chemical And Pharmacological Standardization Of Ashwagandhadi Lehyam: An Ayurvedic Formulation. Journal Of Complementary And Integrative Medicine. Https://Doi.Org/Http://Doi.Org/10.1515/Jcim-2012-0026

[17]. H. Yang, G. Shi, And Q. P. Dou, "The Tumor Proteasome Is A Primary Target For The Natural Anticancer Compound Withaferin A Isolated From 'Indian Winter Cherry,'" Mol. Pharmacol., Vol. 71, No. 2, Pp. 426–437, 2007, Doi: 10.1124/Mol.106.030015.

[18]. S. Srinivasan, R. S. Ranga, R. Burikhanov, S. S. Han, And D. Chendil, "Par-4-Dependent Apoptosis By The Dietary Compound Withaferin A In Prostate Cancer Cells," Cancer Res., Vol. 67, No. 1, Pp. 246–253, 2007, Doi: 10.1158/0008-5472.CAN-06-2430.

[19]. S. D. Stan, E. R. Hahm, R. Warin, And S. V. Singh, "Withaferin A Causes FOXO3a- And Bim-Dependent Apoptosis And Inhibits Growth Of Human Breast Cancer Cells In Vivo," Cancer Res., Vol. 68, No. 18, Pp. 7661–7669, 2008, Doi: 10.1158/0008-5472.CAN-08-1510.

[20]. A. R. Vyas And S. V. Singh, "Molecular Targets And Mechanisms Of Cancer Prevention And Treatment By Withaferin A, A Naturally Occurring Steroidal Lactone," AAPS J., Vol. 16, No. 1, Pp. 1– 10, 2014, Doi: 10.1208/S12248-013-9531-1.

[21]. Y. Cai, Z. Y. Sheng, Y. Chen, And C. Bai, "Effect Of Withaferin A On A549 Cellular Proliferation And Apoptosis In Non-Small Cell Lung Cancer," Asian Pacific J. Cancer Prev., Vol. 15, No. 4, Pp. 1711–1714, 2014, Doi: 10.7314/APJCP.2014.15.4.1711.

[22]. J. T. Thaiparambil Et Al., "Withaferin A Inhibits Breast Cancer Invasion And Metastasis At Sub-Cytotoxic Doses By Inducing Vimentin Disassembly And Serine 56 Phosphorylation," Int. J. Cancer, Vol. 129, No. 11, Pp. 2744–2755, 2011, Doi: 10.1002/Ijc.25938.

[23]. Patil D, Gautam M, Mishra S, Karupothula S, Gairola S, Jadhav S, "Determination Of Withaferin A And Withanolide A In Mice Plasma Using High-Performance Liquid Chromatography_Tandem Mass Spectrometry: Application To Pharmacokinetics After Oral Administration Of Withania Somnifera Aqueous Extract. 2013;80:203–12.," J Pharm Biomed Anal, Vol. 80, Pp. 203–12, 2013, Doi: Https://Doi.Org/10.1016/J.Jpba.2013.03.001.

[24]. E. Hahm, S. Kim, K. B. Singh, K. Singh, And V. Shivendra, "HHS Public Access," Vol. 13, No. 9, Pp. 721–734, 2021, Doi: 10.1158/1940-6207.CAPR-20-0259.A

[25]. Agnihotri AP, Sontakke SD, Thawani VR, Saoji A, Goswami VS. Effects Of Withania Somnifera In Patients Of Schizophrenia: A Randomized, Double Blind, Placebo Controlled Pilot Trial Study. Indian J Pharmacol. 2013 Jul-Aug;45(4):417-8. Doi: 10.4103/0253-7613.115012. PMID: 24014929; PMCID: PMC3757622.

[26]. S. Wankhede, D. Langade, K. Joshi, S. R. Sinha, And S. Bhattacharyya, "Examining The Effect Of Withania Somnifera Supplementation On Muscle Strength And Recovery: A Randomized Controlled Trial," J. Int. Soc. Sports Nutr., Vol. 12, No. 1, Pp. 1–11, 2015, Doi: 10.1186/S12970-015- 0104-9.

[27]. D. Choudhary, S. Bhattacharyya, And S. Bose, "Efficacy And Safety Of Ashwagandha (Withania Somnifera (L.) Dunal) Root Extract In Improving Memory And Cognitive Functions," J. Diet. Suppl., Vol. 14, No. 6, Pp. 599–612, 2017, Doi: 10.1080/19390211.2017.1284970.

[28]. D. L. Palliyaguru, S. V. Singh, And T. W. Kensler, "Withania Somnifera: From Prevention To Treatment Of Cancer," Mol. Nutr. Food Res., Vol. 60, No. 6, Pp. 1342–1353, 2016, Doi: 10.1002/Mnfr.201500756.