

# Apoptotic Activity of Withania Coagulans Methanolic Extract in Cancer Cells Using Rats and Hela Cell Line

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## ABSTRACT

**Background:** The resistance of cancer to conventional therapies has inspired the researchers to search for novel strategies (3). Advances in molecular biology have helped to understand the mechanisms responsible for cell turnover and how tumors evade cell death. By exploring this knowledge, different strategies for treating malignancies have been planned that are based on restoration of natural pathways for cell demise.

**Methods:** Anti cancer and apoptotic role of methanolic extract of withania coagulans was evaluated in this study. The extract was applied on both, the experimental animals in which carcinogens were applied on their skin to introduce cancerous lesions and the hela cell lines. Both responded well.

**Conclusion:** The extracts of Withania coagulans can be used to formulate anti-cancer drugs and can replace many anticancer drugs, which are synthetic in nature and induce immense side effects within the body. Advance studies on this herb will conclude more about its anti-cancerous and apoptotic nature.

**Keywords:** Apoptosis, withania coagulans & cancer cells

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## INTRODUCTION

Cancer is the second commonest cause of death in the world. It is a disease of complex etiology and difficult to treat<sup>1,2</sup>. Cell death is an important component of human physiology which maintains the normal tissue homeostasis. Defects in the cell death mechanisms not only give rise to cancers but also ensure the pathological cell expansion leading to progression of cancer as it also desensitizes cancer cells to immune-mediated attack, radiotherapy and chemotherapy. The resistance of cancers to conventional therapies has inspired the researchers to search for novel strategies<sup>3</sup>. Advances in molecular biology have helped to understand the mechanisms responsible for cell turnover and how tumors evade cell death. By exploring this knowledge, different strategies for treating malignancies have been planned that are based on restoration of natural pathways for cell demise.

The hallmarks of cancer include six biological capabilities acquired during the multistep development of human tumors. These hallmarks constitute an organizing principle for rationalizing the complexities of cancer. Resisting cell death is one of them<sup>4</sup>. The programmed cell death serves as a natural barrier to cancer development. Cell death can proceed by different mechanisms, including apoptosis, necrosis, and autophagy. Apoptosis physiological conditions<sup>5,6</sup>. Several mechanisms by

which cancer cells escape endogenous cell death have been identified, hence identifying how cancer cells achieve selective advantage of survival. Molecules that create the barriers to cell death within tumors have been identified as suitable targets for drug discovery. The main idea is either to restore the integrity of natural pathways for cell turnover or promoting cell death or to induce the activation of the activators of endogenous cell death which are sometimes silenced in cancer cells<sup>7,8</sup>.

**Apoptotic pathway:** The apoptotic machinery is composed of upstream regulators and downstream effector components. The regulators are further divided into two components, one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic pathway e.g., Fas ligand/Fas receptor), and the other sensing and integrating intracellular signals (the intrinsic program). Each initiating activation of protease (caspases 8 and 9, respectively), which internally initiate a cascade of proteolysis involving effector caspases which cause the progressive disassembling and then consumption of cell by the phagocytic cells. The intrinsic apoptotic program is more widely implicated as a barrier to cancer pathogenesis<sup>5</sup>. Cancer cells have negative regulatory effect on caspases and hence suppress apoptosis.

**Withania Coagulans--as an emerging anticancer:** It is often so that the current cancer therapeutic agents severely compromise the normal cellular homeostasis that therapeutic intervention is of limited clinical value. So the aim of any therapeutic strategy should be to target the tumor cells with limited harmful effect to the normal cell function. In current

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years, the naturally occurring compounds and their synthetic analogs have acquired greater attention in the field of cancer research and are promising anti-cancer agents because of their non-toxic and potent anti-cancer properties. Moreover the epidemiological studies have revealed that in those individuals who ingest a large amount of food from plant sources the risk of cancer is decreased<sup>16</sup>. Therefore, identifying anti-cancer compounds in plant extracts has become the major strategy to treat cancers. Nowadays almost 80% of the world population is using plant derived medicine for health improvement because it has no or very less side effects<sup>17</sup>.

Pakistan is very rich in plant resources specially the medicinal ones. Almost 1,000 species of medicinal plants have been reported in the Peshawar region only and 500 species of them are being used for health care practices. Moreover the medicinal plants have mammoth potential but unluckily very little is known about the actual size of production, their capabilities, their conservation status etc. and very little research is carried out in this field so far in Pakistan<sup>18</sup>.

Withania, a small genus of shrubs, is commonly found in Afghanistan, India and southern Pakistan. It is frequently known as panir or vegetable rennet and belongs to Solanaceae family. Withania coagulans is an important medicinal herb as large numbers of phytochemicals have been isolated from it, which are in use in different herbal formulations and pharmaceutical products. Phytochemical analysis of the hydro alcoholic fraction of withania showed the presence of steroids, alkaloids, phenolic compounds, tannins, saponins, carbohydrates, proteins, amino acids and organic acids. On the other hand the chloroform fraction showed the presence of mainly steroids and alkaloids. Pharmacological evaluation has shown the association of these activities with the specific steroidal lactones known as Withanolides present in Withania. The major Withanolides present in Withania somnifera and Withania coagulans are Withaferin A and Withanolide A and Withanone<sup>17,19</sup>.

Withanolides have proved to be potent suppressors of NF-K Beta. This suppression is mediated through inhibition of IKK. This mechanism may account for the ability of Withanolides to suppress the expression of gene products that regulate apoptosis, proliferation, angiogenesis and invasion and hence may prove to be important anticancer agent<sup>17</sup>.

## METHODOLOGY

Study was designed to check the anticancer activity of withania coagulans extract.

**Identification of plant:** Identification of the plant was done by Dr. Tahira Mughal (Prof. of Botany at Lahore College for Women University).

**Extraction procedure:** The plant was dried in the shade after collection and identification. The plant material was ground to powder. Then the powder was placed in the thimble of Soxhelt and extract with methanol at 60°C was collected. The methanol was evaporated under vacuum by rotatory evaporator to get crude methanolic extract.

**Stock solution:** 1 gram of extract was dissolved in 1 ml of DMSO to prepare stock solution of 1000µg/µl then serial dilutions were made (250µg/µl, 100µg/µl, 50µg/µl, 25µg/µl, 10µg/µl, 0.5µg/µl & 0.1µg/µl).

**Experimental: Preparation of rats:** Rats were fed at regular intervals with synthetic rat food (National feeds Lahore) and tap water. Water was given to them by glass bottles fixed with nozzle. On the floor of all rat cages saw dust was spread in two centimeter thickness for the absorption of animal feces and moisture. It also helped to maintain the temperature. Saw dust was changed after 24 hrs. After one week of familiarization the dorsal surface of the albino rats was shaved with electric shaver (area was 5x5cm). Then carcinogens like 7,12-Dimethylbenz (a) anthracene (DMBA) single dose of 100µg/ml/ week for two weeks & 12-O-tetradecanoyl-phorbol-13-acetate (TPA) 10µg/ml twice weekly for 13 weeks after the application of DMBA (both obtained from Sigma Chemical company) were applied for 15 weeks to induce carcinogenesis. Solvents used as dilutors were

1. Acetone: as a vehicle for carcinogens &
2. Methanol: was used in the plant extract.

**Anticancer activity measurement:** Hair loss, appearance of skin lesions such as outgrowth and ulcers were weekly observed, counted and measured with vernier caliper. Skin tissue was also taken by fine needle biopsy after 15 weeks and was examined for atrophy, hyperplasia, Para keratosis and dysplasia. Animals were divided into three study groups

Group A: control group

Group B: dilutors were applied

Group C: different strengths of extract were applied

Group A received no treatment. Group B received application of dilutors and group C received withania coagulans extract as antineoplastic agent twice weekly for next 15 weeks. At the end of the experiment the animals were examined for the gross and microscopic changes in the three different groups.

After the observation of anticancer activity of methanolic extract in cancer cells in rats, the apoptotic activity of the same was assessed in human cancer cell lines.

The Hela cell lines (cervical cancer cell lines) were obtained from Dept. of Applied Zoology. The cells were maintained in the research laboratory of CRIMM, University of Lahore, in 25 cm<sup>2</sup> culture flasks containing Dulbecco's minimal essential media+10% Fetal Bovine Serum in a carbon dioxide incubator (Nuair, USA) at 37°C, in an atmosphere of 5% carbon dioxide.

MTT Assay:

**Short 96 well assay:** Assay was performed in duplicates.

**Day one:**

1. One T-25 flask was trypsinized and 5 ml of complete media was added to these cells. Then cells were centrifuged in a sterile 15 ml falcon tube at 500 rpm in the swinging bucket rotor (~400 x g) for 5 min.
2. Removed media and re-suspended cells to 1.0 ml with complete media.
3. Cells per ml. were counted and recorded aseptically.
4. Cells were diluted with media to 75,000 cells per ml.
5. 100µl of cells were added into each well and incubated overnight.

**Day two:**

6. On day two the cells were treated with withania coagulans extract in different dilutions.

**Day three:**

7. 20µl of 5 mg/ml MTT was added to each well. One set of wells had MTT but no cells (control).
8. Aseptic measures were observed through out the procedure.
9. Then these wells were incubated for 3.5 hours at 37°C C in culture hood.

10. Media was removed carefully so that the cells were not disturbed.
11. 10.150µl MTT solvent was added.
12. Afterwards cells were covered with tinfoil and then were agitated on orbital shaker for 15 min.
13. Absorbance at 590 nm with a reference filter of 620nm was read.

Cell viability was calculated as follows

Relative cell viability calculation=

$$\frac{\text{OD at 570 nm of the sample} \times 100}{\text{OD at 570 nm of Blank}}$$

## RESULTS

Lesions which appeared after the application of DMBA and TPA for 15 weeks in all groups of animals were skin ulcers, loss of hair, scabs and inflammation (Table 1). When methanolic extract of withania coagulans was applied on these lesions for next 15 weeks in group C animals, the lesions cured (Table 4). The control group animals (Table 2) and placebo group b1 & b2 (Table 3) did not show any improvement. The placebo group animals were applied with acetone (group B1) and methanol (group B2). Hence the healing in the group C animals was neither because of acetone nor methanol, the compounds which were used as solvents for carcinogens and withania coagulans extract respectively.

When the same extract was used on Hela cell lines it caused apoptosis of the cancer cell lines even with a concentration of 10 µg/ml. Hence indicating that it has anticancer activity in animal cells as well as human hela cell lines.

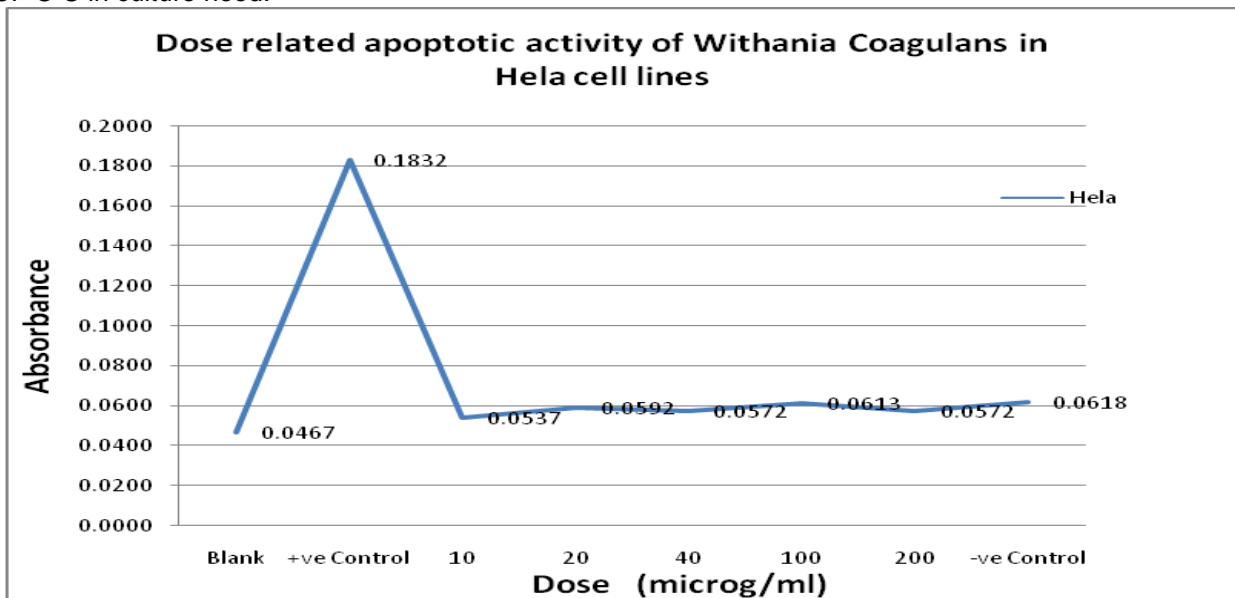


Table 1: Lesion observed in animals (rats) after applying DMBA and TPA for 15 weeks in all groups

Groups	IDN	Sex	Age (wks)	Wt (gm)		Gross Examination					Microscopic Examination		
				Start of Study	End of Study	Ulcer	Lesions		Loss of Hairs	SCB	Epidermis	Dermis	Inflammation
							Number	Size (mm)					
Group A	1	F	6	156	123	++	4	1	Yes	-ve	HP	ED	+ve
	2	F	5	146	99	+++	2	2	Yes	-ve	HP	FB+ED	+ve
	3	M	6	132	101	+++	5	5	Yes	+ve	HP+DP	MFH	+ve
	4	F	6	124	97	+++	1	4	Yes	-ve	HP+DP	FB	-ve
	5	F	5	153	104	++	1	3	Yes	-ve	HP	OT	-ve
	6	M	6	134	101	+	1	2	Yes	-ve	HP+SCI	ED	-ve
	7	F	6	156	113	+++	2	2	Yes	-ve	DP+SCI	FB	-ve
	8	F	6	128	96	++	1	3	Yes	+ve	HP	MFH	+ve
	9	M	5	171	121	+++	1	4	Yes	+ve	HP+DP	ED	+ve
	10	M	6	168	106	++	2	1	Yes	-ve	HP	ED	-ve
Group B1	1	M	5	167	100	++	2	1	No	+ve	HP+DP	FB	+ve
	2	M	6	130	90	++	2	2	Yes	-ve	HP	FB+ED	+ve
	3	M	6	125	95	++	4	5	Yes	+ve	HP	MFH	-ve
	4	M	6	140	100	±	1	3	Yes	+ve	HP+DP	FB	+ve
	5	F	6	160	105	+++	1	3	Yes	-ve	HP+DP	OT	+ve
Group B2	6	F	5	162	109	++	2	1	Yes	+ve	HP+DP	FB	+ve
	7	M	5	155	100	++	1	2	Yes	-ve	HP	FB+ED	-ve
	8	M	6	125	90	+++	1	3	Yes	+ve	HP+DP	MFH	-ve
	9	F	6	140	110	++	2	3	Yes	-ve	HP+DP	FB+OT	+ve
	10	M	6	156	125	+++	1	2	Yes	-ve	HP+DP	OT	-ve
Group C	1	M	5	198	350	+++	2	3	Yes	+ve	HP	FB	+ve
	2	M	6	156	241	++	1	2	Yes	-ve	HP+DP	FB+ED	+ve
	3	M	5	170	363	+	2	3	Yes	+ve	HP+DP	FB	-ve
	4	F	6	145	254	+++	4	1	Yes	-ve	HP+DP	OT	-ve
	5	M	6	156	249	++	3	2	Yes	+ve	HP	FB	+ve
	6	M	5	160	236	+++	2	3	Yes	-ve	HP+SCI	FB+ED	-ve
	7	F	5	140	269	++	2	2	Yes	+ve	HP+DP	FB+ED	-ve
	8	M	5	170	350	+++	1	1	Yes	-ve	HP	MFH	+ve
	9	M	6	150	235	++	1	2	Yes	+ve	HP+SCI	FB+OT	+ve
	10	M	5	155	233	+++	3	2	Yes	-ve	HP	MFH	+ve

IDN= SCB= scabs, HP= ED= edema, FB= fibrosis MFH=OT= SCI=

Table 2: Lesion observed in animals (rats) at the end of 30 weeks previously exposed to DMBA and TPA for 15 weeks in group A (Control)

Groups	IDN	Sex	Age (wks)	Wt (gm)		Gross Examination					Microscopic Examination		
				Start of Study	End of Study	Ulcer	Lesions		Loss of Hairs	SCB	Epidermis	Dermis	Inflammation
							Number	Size (mm)					
Group A	1	F	6	156	123	+++	5	3	Yes	+ve	HP	ED	+ve
	2	F	5	146	99	+++	3	3	Yes	-ve	HP	FB+ED	+ve
	3	M	6	132	101	++++	5	5	Yes	+ve	HP+DP	MFH	+ve
	4	F	6	124	97	++++	3	4	Yes	+ve	HP+DP	FB	+ve
	5	F	5	153	104	+++	4	4	Yes	+ve	HP	OT	+ve
	6	M	6	134	101	++	2	3	Yes	+ve	HP+SCI	ED	+ve
	7	F	6	156	113	+++	3	4	Yes	-ve	DP+SCI	FB	-ve
	8	F	6	128	96	+++	3	5	Yes	+ve	HP	MFH	+ve
	9	M	5	171	121	++++	2	4	Yes	+ve	HP+DP	ED	+ve
	10	M	6	168	106	+++	4	2	Yes	+ve	HP	ED	+ve

Table 3: Lesion Observed in animals (rats) at the end of 30 weeks after application of acetone and methanol previously exposed to DMBA and TPA for 15 weeks in group B (Placebo)

Groups	IDN	Sex	Age (wks)	Wt (gm)		Gross Examination					Microscopic Examination		
				Start of Study	End of Study	Ulcer	Lesions		Loss of Hairs	SCB	Epidermis	Dermis	Inflammation
							Number	Size (mm)					
Group B1	1	M	5	167	100	Nil	Nil	Nil	Yes	+ve	HP+DP	FB	+ve
	2	M	6	130	90	Nil	Nil	Nil	Yes	-ve	HP	FB+ED	+ve
	3	M	6	125	95	Nil	Nil	Nil	NO	-ve	HP	MFH	+ve
	4	M	6	140	100	Nil	Nil	Nil	NO	-ve	HP+DP	FB	+ve
	5	F	6	160	105	+	Nil	Nil	NO	-ve	HP+DP	OT	+ve
Group B2	6	F	5	162	109	Nil	Nil	Nil	NO	-ve	HP+DP	FB	+ve
	7	M	5	155	100	Nil	Nil	Nil	NO	-ve	HP	FB+ED	+ve
	8	M	6	125	90	+	Nil	Nil	NO	-ve	HP+DP	MFH	+ve
	9	F	6	140	110	Nil	Nil	Nil	NO	-ve	HP+DP	FB+OT	+ve
	10	M	6	156	125	Nil	Nil	Nil	NO	-ve	HP+DP	OT	+ve

Table 4: Lesion observed in animals (rats) treated with methanolic extract of withania coagulans at the end of 30 weeks previously exposed to DMBA and TPA for 15 weeks in group C (Study)

Groups	IDN	Sex	Age (wks)	Wt (gm)		Gross Examination					Microscopic Examination		
				Start of Study	End of Study	Ulcer	Lesions		Loss of Hairs	SCB	Epidermis	Dermis	Inflammation
							Number	Size (mm)					
Group C	1	M	5	198	350	+++	2	3	Yes	+ve	HP	ED	+ve
	2	M	6	156	241	++	1	2	Yes	-ve	HP	FB	-ve
	3	M	5	170	363	+	2	3	Yes	+ve	HP	FB	-ve
	4	F	6	145	254	+++	4	1	Yes	-ve	HP	FB	-ve
	5	M	6	156	249	++	3	2	Yes	+ve	HP	ED	-ve
	6	M	5	160	236	+++	2	3	Yes	-ve	HP	ED	-ve
	7	F	5	140	269	++	2	2	Yes	+ve	HP	FB	-ve
	8	M	5	170	350	+++	1	1	Yes	-ve	HP	FB	-ve
	9	M	6	150	235	++	1	2	Yes	+ve	HP	ED	-ve
	10	M	5	155	233	+++	3	2	Yes	-ve	HP	ED	+ve

## DISCUSSION

In our study the methanolic extract of withania coagulans proved to be equally effective in both experimental rats as well as hela cell lines. It has its apoptotic as well as anticancer effect even at very low concentrations. Methanol and acetone were applied on the control groups to exclude their effect on the experimental animals if they were to have any. Moreover methanol was used as control to access its apoptotic effect on hela cell lines. Both proved to be ineffective in rat skin lesions as well as in hela cell lines.

Study would have been more conclusive if we would have studied the intracellular pathway through which it have had caused apoptosis of the hela cell lines. We can extend this study to follow the specific apoptotic pathway associated with withania coagulans extract after doing the separation of different constituents by using HPLC or some other technique.

## CONCLUSION

The present results thus propose that extracts of Withania coagulans can be used to formulate anti-cancer drugs and can replace many anticancer drugs, which are synthetic in nature and induce immense side effects within the body. Further studies are however required to separate and illustrate the active compounds responsible for anti-cancer and apoptotic activity. Advance studies on this herb will conclude more about its anti-cancerous and apoptotic nature.

## REFERENCES

- Sadia Qureshi, M. Zamir Ahmad, Muhammad Tahir Aziz. Serum levels of HER2/neu ECD in women with carcinoma of breast and its correlation with body mass index. JAIME,6:2, 2008, 20-26.
- Sadia Qureshi, M. Sohail Aslam, Qasim Mehmood, Muhammad Tahir Aziz, M. H Qazi Oncolytic viruses-New weapons for cancer therapy. J App Pharm, 2011; 03(03): 320-330.
- Sadia Qureshi, M. Zamir Ahmad, Muhammad Tahir Aziz. Serum levels of Estradiol in women with carcinoma of breast and its correlation with body mass index. JAIME, 4:3: 2008,
- Douglas Hanahan, Robert A. Weinberg Hallmarks of Cancer: The Next Generation Cell, 144: 5, 2011:646-674.
- Danial NN and Korsmeyer SJ. Cell death: critical control points. Cell 116, 2004:205–219.
- Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell, 120, 2005: 159–162.
- Arafat WO. An adenovirus encoding proapoptotic Bax synergistically radiosensitizes malignant glioma. Int J Radiat Oncol Biol Phys 55, 2003:1037–1050.
- Koga S. A novel telomerase-specific gene therapy: gene transfer of caspase-8 utilizing the human telomerase catalytic subunit gene promoter. Hum Gene Ther 11, 2000: 1397–1406.
- Jhon C Reed. Drug Insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat Cli Pra Onc, 3:7,2006:388-398.
- Xiangguo Liu, Ping Yue, Shuzhen Chen, Liping Hu, Sagar Lonial, Fadlo R. Khuri, and Shi-Yong Sun. The Proteasome Inhibitor PS-341 (Bortezomib) Up-Regulates DR5 Expression Leading to Induction of Apoptosis and Enhancement of TRAIL-Induced Apoptosis Despite Up-Regulation of c-FLIP and Survivin Expression in Human NSCLC Cells. Cancer Res,67,2007:4981-4988.
- Pathan N. TUCAN: An anti-apoptotic caspase-associated recruitment domain family protein over-expressed in cancer. J Biol Chem 276, 2001: 32220–32229.
- Kroemer G and Reed JC. Mitochondrial control of cell death. Nat Med 6, 2000: 513–519.
- Evan GI and Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 2001:342–348.
- Fesik SW. Insights into programmed cell death through structural biology. Cell 103,2000: 273–282.
- Adams, J.M., and Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26, 2007:1324.
- Hasmah Abdullah, Azimahtol HL Pihie, Judit Hohmann and Joseph Molnár. A natural compound from Hydrophy tumformicarium induces apoptosis of MCF-7 cells via up-regulation of Bax. Cancer Cell International 2010: 10.
- Deepika Mathur and RC Agrawal. Withenia Coagulans: A Review on the morphological Properties of the shrub. World Journal of Science and Technology 2011: 1(10): 30-37.
- Ali SI, Omer S and Qaiser M. Flora of Pakistan: in Muhammad Afzal and Shehzad A Mufti Natural History research in Pakistan. PASTIC, Islamabad. 2001.
- Atta-Ur-Rehman, Dur-e-Shehwar, Aniq Naz and M Iqbal Ch. Withanoloids from Withenia Coagulans Photochemistry. 2003: 63 (4): 387-90.