



Original Research Article (Experimental)

## Evaluation of apoptotic activity of *Withania coagulans* methanolic extract against human breast cancer and Vero cell lines

Rumana Ahmad <sup>a,\*</sup>, Afreen Fatima <sup>b</sup>, A.N. Srivastava <sup>b</sup>, Mohsin Ali Khan <sup>c</sup><sup>a</sup> Dept. of Biochemistry, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow, 226003, UP, India<sup>b</sup> Dept. of Pathology, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow, 226003, India<sup>c</sup> Chairman Research, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow, 226003, India

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## ABSTRACT

**Background:** The genus *Withania* (Family: Solanaceae) holds an important position in Ayurveda, the Indian traditional system of medicine. *Withania somnifera* Dunal and *Withania coagulans* Dunal have been documented in folklore as panaceas for various ailments since time immemorial. *W. coagulans* (WC), commonly called as Indian cheese maker is used for fermenting milk for cheese production in various parts of India.

**Objectives:** In the study, *in vitro* cytotoxicity of methanolic extract of dried fruits (berries) of WC was evaluated in a dose dependent manner using trypan blue dye exclusion method against human breast cancer cell line MDA-MB-231 and normal kidney epithelial cell line Vero in the range 20–200 µg/ml.

**Material and methods:** The percentage viability of the cell lines was determined by using MTT assay and cytometry.

**Results:** Methanolic extract of WC showed significant anticancer activity against MDA-MB-231 cell line. Cell viability was reduced to about 50% at 40 µg/ml of methanolic extract in 50% DMSO. Cytotoxicity of the extract was lower in 10% and 1% DMSO. On the other hand, methanolic extract of WC did not exhibit any significant *in vitro* activity against Vero cells at 170 and 200 µg/ml. AGE of isolated DNA from treated cancer cells revealed characteristic ladder like fragmentation, a hallmark of apoptosis. HPLC profiling was carried out for identification of the active components, which demonstrated the presence of Withaferin A in the methanolic extract.

**Conclusion:** Methanolic extract of WC possesses apoptotic activity against human breast cancer cells *in vitro* albeit lower in comparison to *W. somnifera* and warrants further investigation.

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## 1. Introduction

Medicines derived from natural products have generated widespread interest and attention globally on account of their safety, efficacy and relatively fewer side effects. This has caused a paradigm shift towards opting for safer plant-based remedies. The plant, *Withania coagulans* Dunal is well-documented in Ayurveda as a cure for a plethora of diseases and conditions [1]. Fruits and leaves of *W. coagulans* Dunal (Fig. 1) contain an enzyme called Withanin and are commonly used to coagulate milk [1,2].

Various parts of this plant are known to possess and exhibit a wide spectrum of biological activities [1]. The fruit is a berry having a sweet taste with reported sedative, emetic and diuretic properties [2]. The shrub has a widespread distribution in India and is found in Punjab, Rajasthan, Simla, Kumaun and Garhwal regions [1]. The dry fruits are commonly used for treatment and management of diabetes in northern parts of India [1]. No wonder, the fruit is also commonly referred to as 'tukhm-e-hayat' (fruit of life). The plant has also been found to possess a range of activities viz. hypoglycemic [3,4], hypolipidemic, free radical scavenging, cardiovascular, hepatoprotective, anti-inflammatory, wound healing, antitumor, immuno-suppressive, cytotoxic, antifungal and antibacterial properties [1].

The most active chemical components of genus *Withania* are withanolides, which are known anticancer agents, as well as flavonoids [2]. The detailed mechanism underlying the anti-cancer effect of WC is still largely unknown. The anti-mutagenic activity of

\* Corresponding author.

E-mail address: [rumana\\_ahmad@yahoo.co.in](mailto:rumana_ahmad@yahoo.co.in) (R. Ahmad).

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Fig. 1. (a). *Withania coagulans* (L.) Dunal Habit (b) Fruits (Image courtesy Gupta, 2012).

WC fruit extracts has been evaluated in bone marrow cells of mice. The results showed that a single i.p. dose of 500–1500 mg/kg body weight of WC fruit extract before 24 h, significantly reduced micronucleus formation in mouse bone marrow cells as compared to cyclophosphamide group [5].

Withaferin A, Withanolide A and Withanone are the major withanolides present in genus *Withania* [6,7] out of which Withaferin A is the most important in terms of its concentration and spectrum of activity [1]. It possesses significant antibiotic activity and its anti-tumor effect has been studied against malignant nasopharyngeal (KB) cells *in vitro* [1]. It interferes in cell division by arresting mitosis at metaphase. *In vivo* studies have also demonstrated the growth-inhibitory and radio-sensitizing effects of Withaferin A in mouse Ehrlich ascites carcinoma [8,9]. It has also been found to arrest cell division in embryonal chicken fibroblast cells. Anti-cancer and apoptotic activities of WC aqueous and methanolic fruit extracts have also been studied on DMBA induced skin papillomagenesis *in vivo* [5] as well as *in vitro* [10,11]. In the present study, the apoptotic activity of WC has been tested against human breast cancer as well as normal epithelial cell lines. The results revealed that the methanolic extract of fruits possesses significant activity against breast cancer cell line but no appreciable activity against the normal cell line. The genus *Withania* can, as such, be used as an adjunct or complementary medicine in patients undergoing aggressive treatment in clinical settings against a number of chronic and debilitating diseases like cancer.

## 2. Materials and methods

### 2.1. Reagents

PBS (pH = 7.2, 1x), 0.25% trypsin–EDTA (1x), Dulbecco's Modified Eagle's Medium DMEM/F-12 (1x), 0.4% trypan blue, and antibiotic/antimycotic solution (100×) were obtained from Gibco, Life Technologies; whereas fetal bovine serum (FBS) and MTT were obtained from Himedia. Doxorubicin hydrochloride solution was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dimethyl sulfoxide (DMSO) from Calbiochem. Withaferin A standard was from Natural Remedies Ltd. Veerasandra, Bangalore-100. All HPLC grade reagents were used in HPLC. All other chemicals were of analytical grade.

### 2.2. Collection of plant material

The plant WC was identified by a competent botanist from National Botanical Research Institute (NBRI), Lucknow. The dry fruits

of *W. coagulans* were purchased from the local market of Lucknow, India, and were shade dried further followed by grinding.

### 2.3. Sample preparation

All procedures were carried out as per our previous study reporting the cytotoxic activity of methanolic and ethanolic extracts of *Withania somnifera* [12]. Briefly, 50% methanolic extract was prepared by extracting 25 g of powder of WC berries thrice. All three extracts were pooled and filtered through Whatman No. 1 filter paper (125 mm) and concentrated in a water bath till formation of a semi-solid paste. The paste was dried in a desiccator until a powdered form was attained and stored in an air-tight container till further use. For comparing better dissolution and activity, 20 mg/ml each of the powdered extract was dissolved in 1%, 10%, and 50% DMSO. All extracts were filtered through sterile syringe filter units (0.22- $\mu$ m, Millipore, Fisher Scientific) before addition to cell culture medium.

### 2.4. Biological evaluation

#### 2.4.1. Cell lines

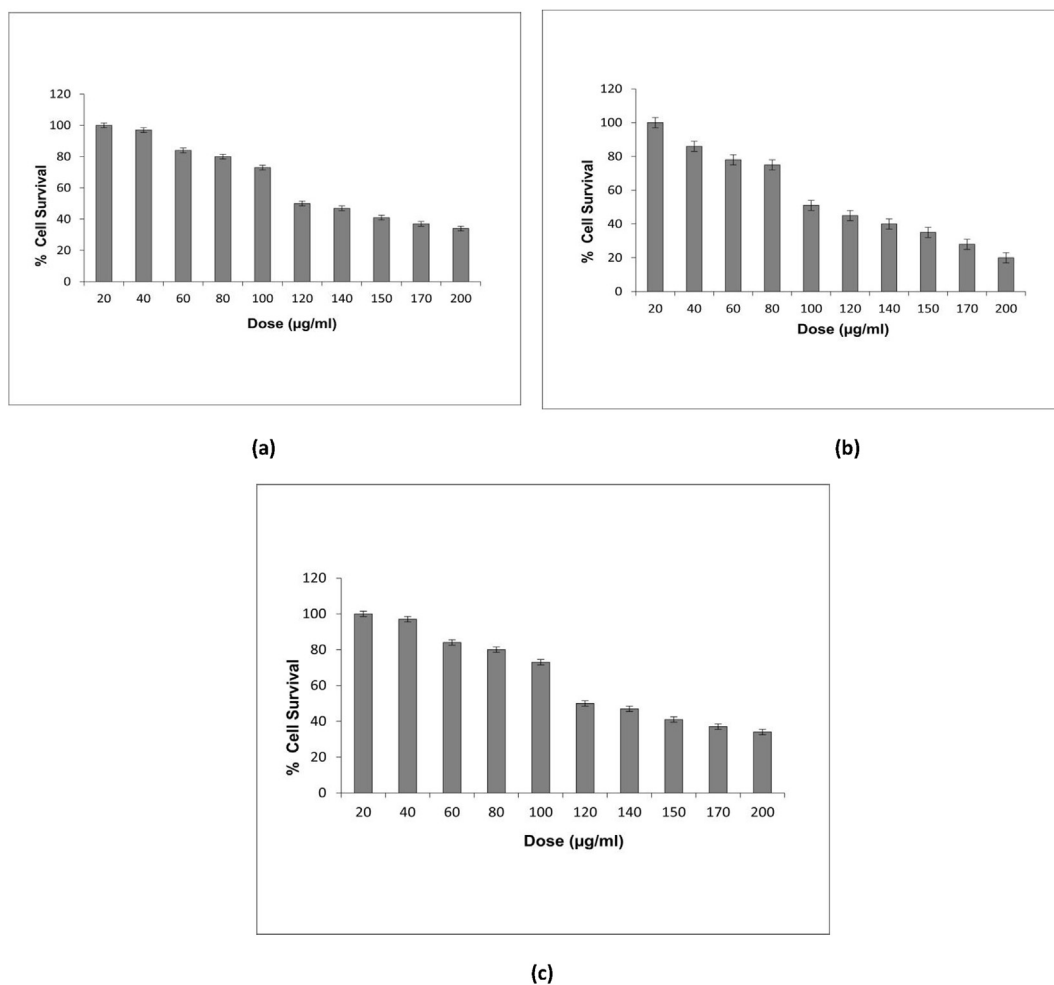
Two cell lines viz. MDA-MB-231 (human breast carcinoma) and Vero (normal African green monkey kidney epithelial cells; ATCCCL-81), were obtained from the National Centre for Cell Science (NCCS), Pune, India, and as such, were maintained in Tissue and Cell Culture Lab, Era's Lucknow Medical College, Lucknow, as per previously established protocol [12].

### 2.5. Cell culture

For the experiments, cells were trypsinized and seeded at a density of  $0.5 \times 10^5$  cells/well for 24 h in 6-well plates (Linbro, MP Biomedicals) for adherence. Cells were exposed to 20–200  $\mu$ g/ml of methanolic extract of WC in DMSO for the next 48 h. Suitable untreated controls were also included. All experiments were done in triplicates. Results were plotted as cell viability versus time period graph.

### 2.6. Morphological study

Cells were observed and photographed for morphological characteristics under a phase contrast microscope (Nikon Eclipse Ti, Japan).



**Fig. 2.** Dose dependent effect of WC methanolic extract in 1% (a) 10% (b) and 50% (c) DMSO on viability of MDA cells *in vitro*. Final concentration of DMSO in each well did not exceed 0.5% (v/v).

## 2.7. Cytotoxicity assays

### 2.7.1. Trypan blue dye exclusion assay

The assay was carried out as reported previously [12].

### 2.7.2. (Methyl Tetrazolium-MTT assay) Determination of optimal cell number for the assay

For standardization, serial dilutions of MDA-MB-231 cell line were carried out/100 µl in 96-well microtiter tissue culture plates (Linbro, MP Biomedicals). MTT was performed as per published protocol [12]. Absorbance values were recorded in a Biorad PW41 ELISA plate reader at 550 nm with a reference wavelength of 630 nm and interpreted as cell number versus absorbance graph.

### 2.7.3. Evaluation of cytotoxicity and cell viability

Treated and control wells were subjected to MTT assay by appropriate dilution such that the final concentration of DMSO in each wells was <0.5% (v/v). Briefly, MDA cells were seeded at a frequency of 16,000/100 µl in 96-well microtiter tissue culture plates initially for 24 h and subsequently after addition of WC extract (20–200 µg/ml in 1%, 10% and 50% DMSO), for another 48 h. Wells containing medium only served as blanks. Doxorubicin hydrochloride, was used as a positive control. Cells in medium

containing vehicle (1%, 10% and 50% DMSO) served as controls. Results were interpreted as percentage cell viability obtained by plotting absorbance against the concentration of the extract in µg/ml. The % cell viability was calculated as  $\{(A_T - A_B)/(A_C - A_B)\} \times 100$  where,

$A_T$  = Absorbance of the treatment well

$A_B$  = Absorbance of the blank

$A_C$  = Absorbance of the control well

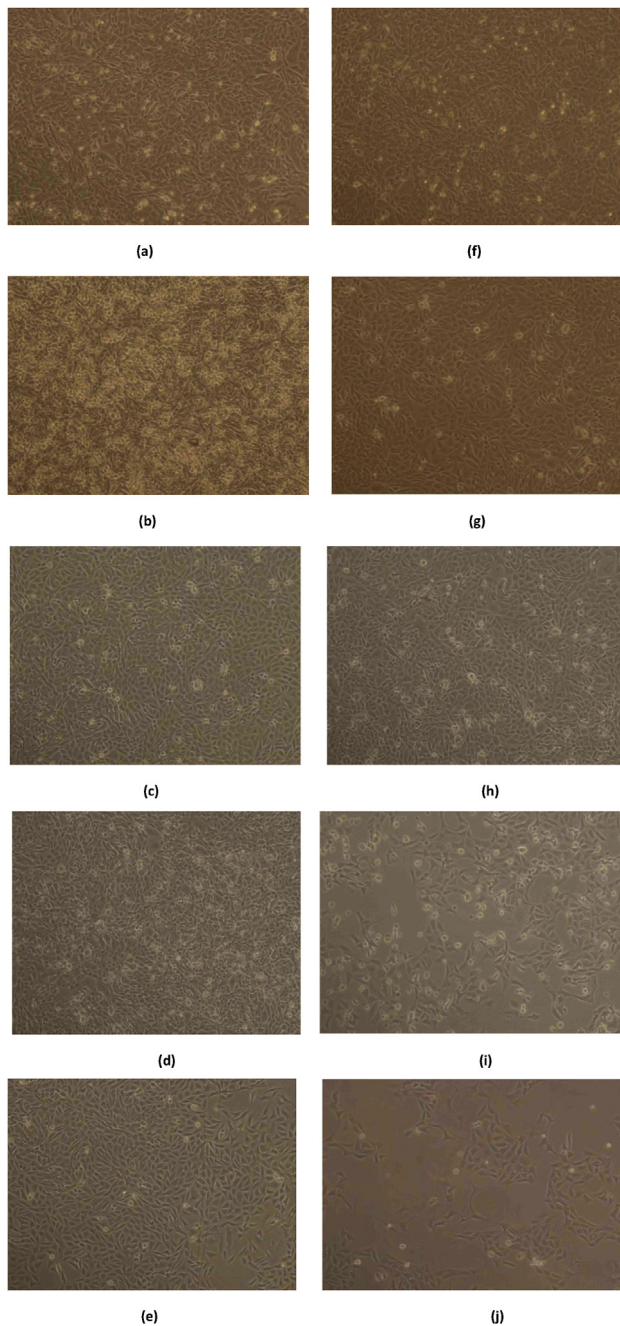
% cell inhibition = 100 – Cell Survival

The IC<sub>50</sub> values of the extracts were determined from the plot as the concentration which decreased the cell viability by 50%.

### 2.7.4. Comparison of the cytotoxic activity of extracts

Dose dependent effect of WC fruit extract on human breast cancer cells was compared to that on normal epithelial cells (Vero) using MTT assay as described above. Briefly, Vero cells were seeded (14,000/100 µl) in 96-well microtiter tissue culture well plates initially for 24 h and then treated with WC extract at two concentrations (170 and 200 µg/ml) found to be cytotoxic to the cancer cells, for the next 48 h followed by MTT assay. Results were observed, recorded and interpreted as discussed above.





**Fig. 3.** (a–e) Controls showing untreated MDA human breast cancer cells in 50% DMSO and (f–j) the cytotoxic activity of WC methanolic extract at 20, 60, 100, 170 and 200 µg/ml, respectively, in 50% DMSO after 48 h (magnification 10 $\times$ ). The final concentration of DMSO in each well did not exceed 0.5% (v/v).

## 2.8. HPLC analysis

### 2.8.1. Preparation of the standard for HPLC analysis

Withaferin-A standard was dissolved in HPLC grade methanol (10 mg/50 ml) and diluted 50 times in methanol. Linearity was observed for Withaferin-A assay in the range of 0.2–20 µg/ml.

### 2.8.2. Sample preparation

WC extract was made at a concentration of 1.0 mg/ml in 0.5% of HPLC grade DMSO and diluted 50 times in HPLC grade methanol.

Sample and Withaferin-A solutions were filtered through sterile 0.45 µm Millipore filters prior to injection. The injection volume was 10 µl for both standard and sample.

### 2.8.3. Procedure

HPLC was performed on a Waters Alliance 515 HPLC system, equipped with two Waters 515 pumps, a Waters Pump Control Module, degasser, injector, and a Waters 2998 photodiode array detector (Waters, Milford, MA, USA) as reported previously [12]. For separations, gradient elution of mobile phase was applied for 55 min as reported earlier [12].

## 2.9. DNA isolation from treated and control cells

At the end of treatment (48 h), adherent cells were trypsinized, pooled with suspended dead cells, centrifuged and finally suspended in PBS. DNA was isolated using NucleoSpin Blood Kit (Machery-Nagel, Germany).

## 2.10. DNA fragmentation assay

Agarose gel electrophoresis (AGE) of isolated DNA from treated cells was carried out on a 1.5% gel at 60 V for 90 min using 1x TBE buffer in a Genei electrophoresis unit (Bengaluru, India). Gels were stained with ethidium bromide and DNA was visualized in a gel documentation system (Biorad, USA).

## 2.11. Data interpretation and statistical analysis

Absorbance values lower than the controls indicated a reduced rate of cell proliferation in MTT assay. Conversely, a higher absorbance corresponded to increased cell proliferation. Evidence of cell death was inferred from morphological analysis as dead cells appeared as floating, rounded cells, as compared to the adherent spindle-shaped live cells. Results were expressed as mean  $\pm$  SD of the experiments done in triplicates.

## 3. Results

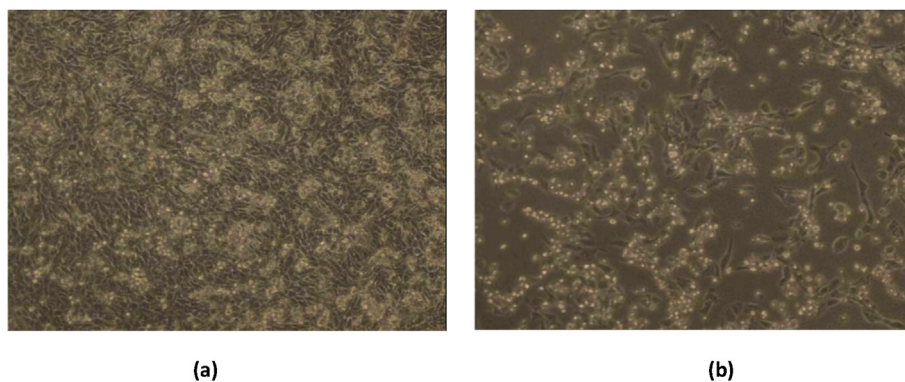
### 3.1. Methanolic extract of WC showed cytotoxicity against human breast cancers cells with less effect on normal cells

It was found that WC methanol extract in 50% DMSO was the most effective in causing cytotoxicity in MDA cells (IC<sub>50</sub> value of approx. 40 µg/ml) as compared to WC extracts in 1% and 10% DMSO. Fig. 2 depicts the dose dependent effect of methanolic WC extract in 1%, 10% and 50% DMSO on MDA cells using MTT assay.

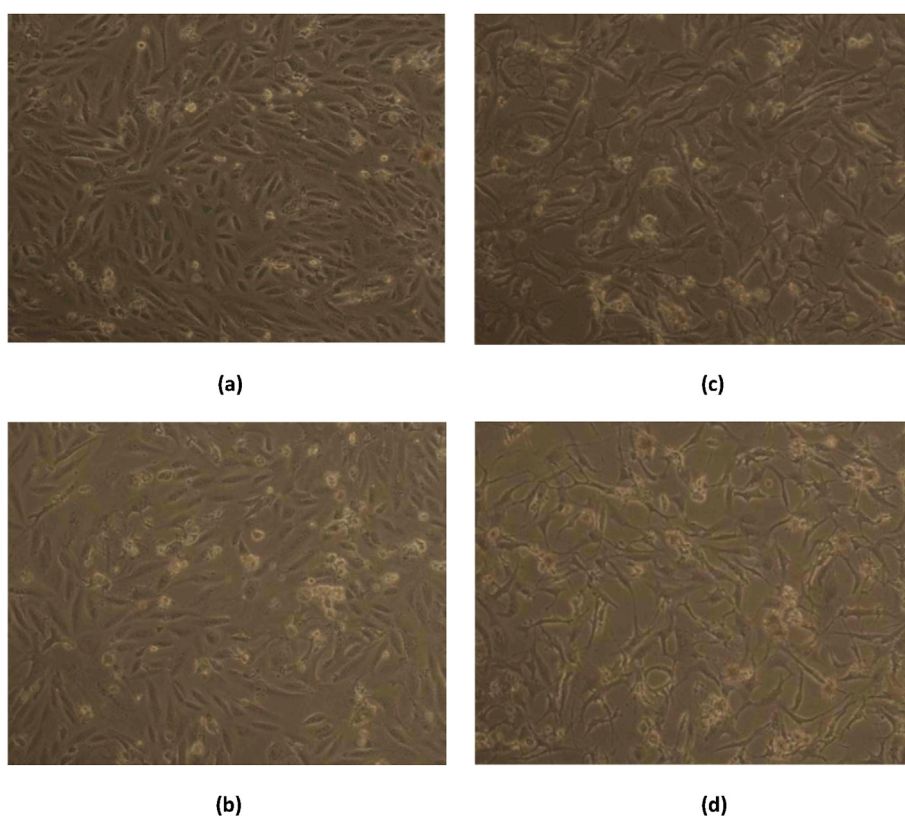
Fig. 3 depicts the morphological analysis of untreated versus treated cells with respect to WC methanolic extract (20–200 µg/ml). It is evident from the figures that WC showed cytotoxic as well as cytostatic effect on human breast cancer cells MDA-MB-231 at higher concentrations (> 60 µg/ml). Methanolic extract had an IC<sub>50</sub> of 120 and 51 µg/ml in 1% and 10% DMSO, respectively, as compared to 40 µg/ml in 50% DMSO. The treated cells displayed an altered morphology under inverted microscope.

Methanolic extract did not show any significant activity when 1% DMSO was used as a vehicle. 50% DMSO proved to be a better vehicle for dissolution of the WC extract in comparison to 1% and 10% DMSO. DMSO at concentrations of either 0.25% or 0.5% did not have any cytotoxic effect of its own (Figs. 3–5). Doxorubicin exhibited cytotoxic and dose dependent inhibition of cell proliferation and the IC<sub>50</sub> value of doxorubicin against MDA-MB-231 cells was determined as 0.50  $\pm$  0.03 µM (Fig. 4).

The effect of WC methanolic extract was also tested on Vero cells, an African green monkey kidney epithelial cell line (Fig. 5).



**Fig. 4.** (a) Control showing untreated MDA human breast cancer cells in presence of 0.5% DMSO and (b) Cytotoxic activity of doxorubicin chloride at 0.5  $\mu$ M in 0.5% DMSO after 48 h (Magnification 10 $\times$ ).



**Fig. 5.** (a–b) Controls showing untreated Vero human kidney epithelial cells in 50% DMSO (c–d) Effect of WC methanolic extract at 170 and 200  $\mu$ g/ml respectively in 50% DMSO after 48 h (Magnification 10 $\times$ ). Final concentration of DMSO in each well did not exceed 0.5% (v/v).

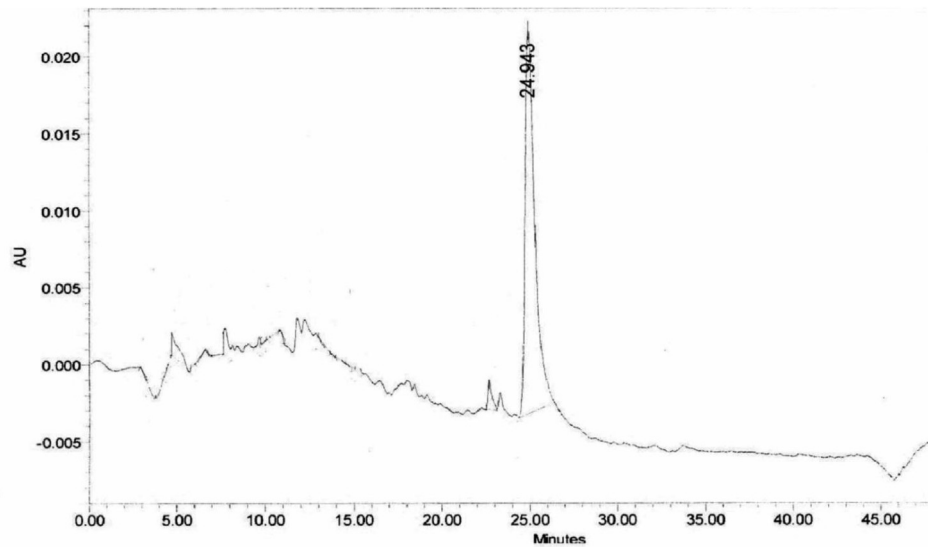
The extract did not show any significant cytotoxic effect on the normal cell line at concentrations that were cytotoxic to human breast cancer cells (170 and 200  $\mu$ g/ml) (Fig. 3).

Qualitative phytochemical characterization of extract constituents was carried out using HPLC (Fig. 6a, b). Withaferin-A was detected in the extract ( $R_t = 24.9$  min), albeit in small amounts thus, explaining the low activity of the WC extract versus *W. somnifera* (WS) methanolic extract as reported earlier [12]. The analytical HPLC method used in the study provided a good baseline resolution of peaks of withanolides present in WS extracts with reference to Withaferin-A standard ( $R_t = 24.9$  min). DNA isolated from WC-treated cells showed characteristic DNA ladder formation

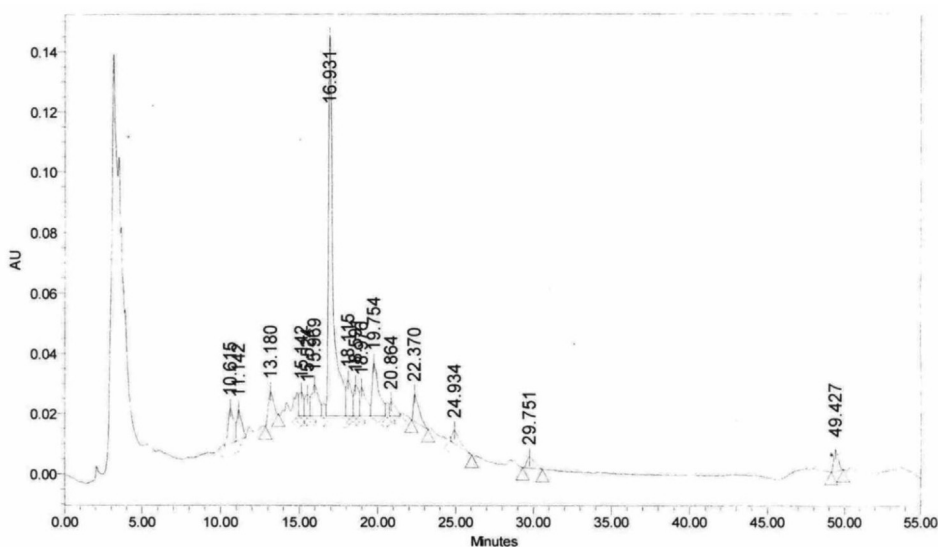
on AGE (Fig. 7), further corroborating the fact that the methanolic extract of WC induced apoptosis in breast cancer cells [13].

#### 4. Discussion

The advantages of using herbal remedies over synthetic chemotherapeutic drugs are manifold, more so because synthetic agents severely compromise the normal cellular homeostasis and often, the therapeutic intervention afforded is of limited clinical significance. So the aim of any therapeutic strategy should be selective targeting of cancer/tumor cells with minimal harm to normal cells. In recent years, compounds derived from natural



(a)



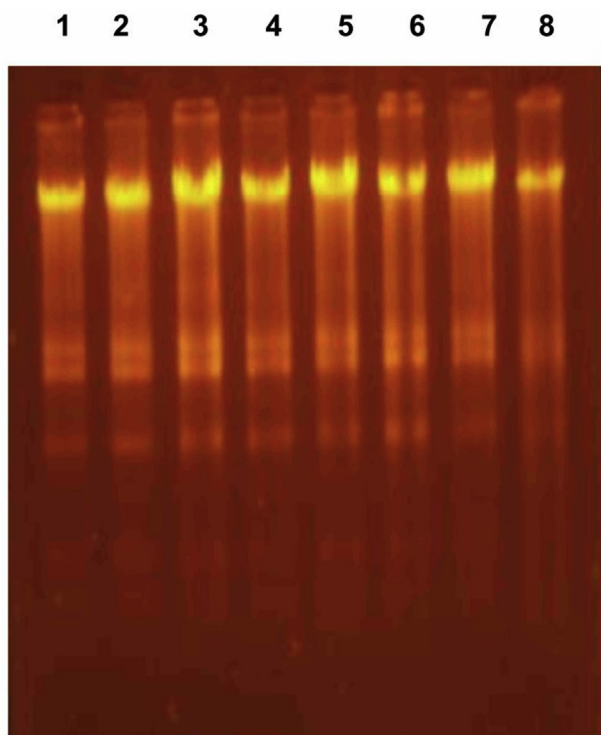
(b)

**Fig. 6.** (a) HPLC profile of Withaferin-A working standard. Withaferin-A, ( $R_t = 24.943$  min) (b) HPLC profile of methanolic extract of WC fruits under identical conditions. Withaferin-A, ( $R_t = 24.934$  min).

products as well as their man-made counterparts have achieved greater prominence and preference in the area of cancer therapy and hold good potential as anticancer agents on account of their safety, potency and efficacy [10]. A number of epidemiological studies have revealed that most vegetarians and vegans report less incidence of cancer [14]. Thus, evaluation of plant extracts for identification and presence of anticancer compounds in terms of their potency and therapeutic index has become the mainstay to treat cancers. Nowadays, almost 80% of the medicine being used globally for various health purposes is plant-derived on account of it having no or very little side effects [15].

In our previous study reporting the cytotoxic activity of methanolic and ethanolic extract of *W. somnifera*, the methanolic extract of WS was found to possess an  $IC_{50}$  of 40 and 30  $\mu\text{g/ml}$  in 10% and 50% DMSO respectively [12] as compared to  $IC_{50}$  values of 51 and 40  $\mu\text{g/ml}$  in 10% and 50% DMSO respectively, obtained for the WC extract in the current study. Withaferins are the main active components of genus *Withania*. For comparative purpose, the content of Withaferin-A in methanolic extract of WS was found to be higher as compared to methanolic extract of WC, thus accounting for the higher anticancer activity (lower  $IC_{50}$ ) of the WS methanolic extract as compared to WC methanolic one.





**Fig. 7.** DNA fragmentation assay for detection of apoptosis. Gel Electrophoresis of DNA isolated from total cells (live + dead) in control and treated flasks. Lane 1–8: DNA from cells treated with 60, 80, 100, 120, 140, 150, 170 and 200 µg/ml WC extract in 50% DMSO, respectively. WC induced DNA ladder formation, a hallmark property of apoptosis. The data are representative of three independent experiments.

## 5. Conclusion

The fruit extracts of *W. coagulans* can be used to formulate novel drugs in future or might be used as adjunct/complementary therapy given along with the main line of treatment. Advanced studies on this plant would unravel the detailed mechanism behind its anticancer and apoptotic activity.

## Compliance with ethical standards

No permission was sought from the Institutional Ethics Committee, Era's Lucknow Medical College, Lucknow, as the work did not involve human or animal subjects.

## Conflict of interest

The authors declare no conflict of interest.

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## References

- [1] Gupta V, Keshari BB. *Withania coagulans* Dunal (Paneer Doda): a review. Int J Ayurvedic Herb Med 2013;3:1330–6.
- [2] Gupta PC. *Withania coagulans* Dunal-an overview. Int J Pharm Sci Rev Res 2012;12:68–71.
- [3] Alam A, Siddiqui MY, Hakim MH. Clinical efficacy of *Withania coagulans* Dunal and *Trigonella foenum-graecum* Linn. in type 2 diabetes mellitus. Ind J Trad Knowl 2009;8:405–9.
- [4] Ojha S, Alkaabi J, Amir N, Sheikh A, Agil A, Fahim MA, et al. *Withania coagulans* fruit extract reduces oxidative stress and inflammation in kidneys of streptozotocin-induced diabetic rats. Oxidative Med Cell Long 2014;2014:201436.
- [5] Mathur D, Agrawal RC. Anticarcinogenic potential of *Withania coagulans* fruit against skin papillomagenesis in Swiss albino mice. Rec Res Sci Tech 2013;5:1–4.
- [6] Jain R, Kachhwaha S, Kothari SL. Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and *Withania coagulans*: a review. J Med Plants Res 2012;6:5388–99.
- [7] Uddin Q, Samiulla L, Singh VK, Jamil SS. Phytochemical and pharmacological profile of *Withania somnifera* Dunal: a review. J Appl Pharm Sci 2012;2:170–5.
- [8] Devi PU, Sharada AC, Solomon FE. *In vivo* growth inhibitory and radiosensitizing effects of Withaferin A on mouse Ehrlich ascites carcinoma. Cancer Lett 1995;95:189–93.
- [9] Sharada AC, Solomon FE, Devi PU, Udupa N, Srinivasan KK. Antitumour and radiosensitizing effects of Withaferin A on mouse Ehrlich ascites carcinoma *in vivo*. Acta Oncol 1996;35:95–100.
- [10] Qureshi S, Aziz MT, Qazi MH, Saqib T. Apoptotic activity of *Withania coagulans* methanolic extract in cancer cells using rats and HeLa cell line. Pak J Med Health Sci 2015;9:123–7.
- [11] Maqsood M, Qureshi R, Ikram M, Ali S, Rafi M, Khan JA, et al. Preliminary screening of methanolic plant extracts against human rhabdomyosarcoma cell line from salt range. Pak J Bot 2015;47:353–7.
- [12] Srivastava AN, Ahmad R, Khan MA. Evaluation of *in vitro* cytotoxic activity of *Withania somnifera* extracts against MDA-MB-231 and Vero Cell Lines. Sci Pharm 2016;84:41–59.
- [13] Maliyakkal N, Udupa N, Pai KSR, Rangarajan A. Cytotoxic and apoptotic activities of extracts of *Withania somnifera* and *Tinospora cordifolia* in human breast cancer cells. Int J Appl Res Nat Prod 2013;6:1–10.
- [14] Abdullah H, Pihie AHL, Hohmann J, Molnár J. A natural compound from *Hydnophytum formicarum* induces apoptosis of MCF-7 cells via up-regulation of Bax. Cancer Cell Int 2010;10.
- [15] Mathur D, Agrawal RC. *Withania coagulans*: a review on the morphological properties of the shrub. World J Sci Tech 2011;1:30–7.