

Evaluation of *In Vitro* Cytotoxic Activity of Ethanolic Extract of *Azadiracta indica* Leaves as a Function of pH on Human Breast Cancer Cell Line MDA-MB 231

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ABSTRACT

Cancer is one of the leading causes of mortality and morbidity in the world and in US, breast cancer is the second most common cancer in women after skin cancer. Researchers have developed various strategies for combating and treating cancer viz. surgery, chemotherapy, radiotherapy as well as plant derived and plant based drugs. Phytotherapeutic agents have no serious side effects and they are better assimilated than their synthetic counterparts. *Azadiracta indica* (AI) commonly known as 'Neem' in India, has many therapeutic properties viz. anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antiulcerogenic, antifungal, antibacterial as well as antitumor. The antineoplastic property of AI is due to its apoptosis-inducing, antiangiogenic, and immunomodulatory effects via several molecular mechanisms. In the present study, individual as well as combined effect of ethanolic extract of AI and pH has been evaluated on survival and viability of human breast cancer cell line MDA-MB-231. It was found that a combination of low pH (6.2) and AI extract (1600 µg/ml) caused significant mortality (95.7%) in the breast cancer cell line MDA-MB-231. The IC₅₀ value for AI extract at pH 7.1 was found to be 200 µg/ml. The combined cytotoxicity at acidic pH in presence of AI extract was compared to that caused only in presence of AI. It was found that low pH potentiates the cytotoxic effect of AI, although

independently, low pH promotes cancer cell survival and viability. Such a study regarding anti-proliferative activity of AI as function of pH has not been reported before and warrants further and future investigation.

Key words: *Azadiracta indica*, pH, cytotoxicity, MDA-MB-231

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INTRODUCTION

Of all the diseases afflicting mankind, cancer is perhaps the deadliest and the most debilitating. An estimated diagnosis of 1,688,780 new cancer cases and about 600,920 cancer-related deaths in the US^[1] is predicted in 2017. Breast cancer is the second most common cancer in women after skin cancer in the US. It can occur in both men and women, but it is rare in men. In 2017, it is predicted that there will be an estimated 255,180 new breast cancer cases (both female and male) diagnosed and 41,070 estimated breast cancer deaths in the US.^[2,3]

As per the National Cancer Registry Programme (NCRP), and National Institute of Cancer Prevention and Research (NICPR), breast cancer is the most common cancer in women in India and accounts for 27% of all cancers in women.^[4,5] As per Globocan data; 1,44,937 new breast cancer cases were registered in 2012 and there were 70,218 deaths due to breast cancer. Overall, 1 in 28 women is likely to develop breast cancer during her lifetime. The incidence rate is more in urban areas versus rural areas.^[6]

Metastatic breast cancer cells can get into the blood or lymph system and be carried to other parts of the body viz. the lungs, liver, bones or brain where they form small tumors (micro-metastases).^[7] This is classified as stage 4 of breast cancer. Causes of breast cancer can be life-style driven (habits, obesity, diet) or genetic (BRCA1 and BRCA2) as well as both. Presently, there is still no effective cure for breast cancer. Researchers and scientists are working towards finding novel and effective therapies for cancer.^[8] Synthetic hormone therapy drugs viz. tamoxifen and raloxifene have limited use for breast cancer treatment because of their side effects. Other drugs viz. bisphosphonates (drugs for osteoporosis), non-steroidal anti-inflammatory drugs (NSAIDs), statins (cholesterol-lowering) and, particularly enzyme inhibitors such as exemestane, anastrozole and letrozole (inhibit aromatase) and COX-2 inhibitors are presently being evaluated in clinical trials for breast cancer reduction risk.^[9]

Plants are the preferred choice for the search and discovery of novel

cancer drug leads by virtue of their containing a staggering array of phytoconstituents having diverse structures and belonging to different classes, most of which have no proven or known side effects. Another advantage of developing phototherapeutic drugs for cancer is the relatively low cost as compared to drug synthesis in a chemical lab.^[10]

In neoplasia, there is alteration in the way a normal cell divides. Under normal conditions, a defective or damaged cell undergoes apoptosis. But in cancer, the defective cell becomes resistant to apoptosis and does not die and continues to divide uncontrollably. Hence, the prerequisite for breast cancer therapy is that the defective cancer cells are induced to undergo apoptosis by either physical or chemical means or a combination of both. In the present study, cytotoxic and proapoptotic effect of a combination of low pH and ethanolic extract of AI leaves was studied on human breast cancer cell line MDA-MB-231. AI is well known medicinal plant in India and neighboring countries and is also the most versatile medicinal plant having a wide spectrum of biological activities. Every part of the tree has been used as a household remedy for numerous ailments, since time immemorial.^[11-16]

The antineoplastic potential of AI has garnered much attention as well as interest in the last few years.^[17,18] Anti-proliferative potential of aqueous as well as alcoholic extracts of AI has been tested on a number of cancer cell lines *in vitro* and have the ability to act as a therapeutic agents for combating cancers.^[19,20] In the present study it was found that

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ethanolic extract of AI leaves in presence of low pH had a significant apoptotic effect on breast cancer cells as compared to breast cancer cell survival at low pH in isolation.

MATERIALS AND METHODS

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 (100X) were obtained from Gibco, Life Technologies; whereas fetal bovine serum (FBS) and MTT were obtained from Himedia. Dimethyl sulfoxide (DMSO) was from Calbiochem. All other chemicals were of analytical grade.

Ethanolic extract preparation of AI leaves

Fresh AI leaves were collected from the area around Sarfarazganj, Hardoi Road, Lucknow, washed with distilled water and dried in the sun. Approximately, 100 g dried leaves were weighed and grinded into a fine powder using a blender and suspended in 80% ethanol. After 24 h, the supernatant was collected and the residue was re-suspended in fresh solvent. The same procedure was repeated twice. All supernatants were collected, pooled and evaporated in a water bath at 100°C until a semi-solid paste was obtained. The extract was dried in a desiccator and stored in air tight bottles. For cell culture studies, an 80 mg/ml extract in 1% DMSO was prepared. The extract was sterilized using 0.22 µ Axiva filters.

Cell culture

The cell line was maintained by serial passaging in 25 cm² flasks as reported previously.^[18] Briefly, MDA-MB-231 cells were seeded in 6-well plates at a density of 5 × 10⁴ cells/ml and were maintained in medium with varying pH viz. 6.2, 6.5, 6.8, 7.1 and 7.4. The plates were kept in a 5% CO₂ incubator maintained at 37°C for 24 h. After 24 h, the wells were treated as follows: The five experimental wells corresponding to five sets of pH values mentioned above received AI extract at the rate of 400, 600, 800 and 1600 µg/ml each in 1% DMSO. The corresponding control wells of five sets of pH values (containing cells maintained in medium with varying pH viz. 6.2, 6.5, 6.8, 7.1 and 7.4, respectively) received an equal amount of vehicle (1% DMSO) after 24 h. The cells were incubated and observed for 48 h.

Morphological analysis

Cells were visualized and photographed after 24 and 48 h using a phase contrast microscope (Nikon Eclipse TS100) under 10X and 40X magnification.

Cytotoxicity assays

a. Trypan blue dye exclusion assay

The assay was carried out as reported previously.^[18]

b. Cytometer based analysis

For cell viability analysis, MDA cells from experimental and control wells were trypsinized and resuspended in culture medium. Propidium iodide was added and cells were incubated at RT for 5 min and counted in a Tali Image-Based Cytometer, Life Technologies (Invitrogen). The number of live and dead cells in experimental and control wells was determined. The number of live cells in both experimental and control wells was used for calculating the percentage cytotoxicity as % Cytotoxicity=Live Cell No. in Treated Wells- Live Cell No. in Control Wells/Live Cell No. in Control Wells × 100).

Statistical analysis

Results were expressed as mean ± SD of experiments done in triplicates.

RESULTS

Table 1 depicts the combined effect of pH and AI ethanolic extract (1600 µg/ml) on MDA-MB-231 cells. When only pH was used as determinant of cytotoxicity, it was found that pH 7.4 had maximum cytotoxicity (11.2%) as reported previously.^[18] The results were found to be in agreement with previously reported values as alkaline pH is toxic to cancer cells. However, when AI extract was included in the study as a function of pH, it was found that AI extract worked best in an acidic medium (pH 6.2). The pH of AI extract has been previously reported to be acidic.^[21] This means that AI extract has better cytotoxicity in an acidic medium. The percentage cytotoxicity of cells increased significantly (95.7%) in presence of AI extract at pH 6.2 versus 0.96% in pH 6.2 independently.

Table 2 and Figures 1-5 respectively depict the dose dependent effect of AI ethanolic extract in the range 400-1600 µg/ml on MDA-MB-231 cells at pH values ranging from 6.2-7.4. It was seen that AI ethanolic extract at 1600 µg/ml had maximum cytotoxicity (95.7%) on MDA cells when pH of medium was maintained at 6.2. The IC₅₀ value of the extract was found to be 200 µg/ml at pH 7.1. Dead cells appeared as floating, rounded cells, as compared to the adherent spindle-shaped live cells [Figures 1-5 E-H].

DISCUSSION

Since, the extracellular pH of tumors is generally more acidic than normal tissue,^[22,23] due to the consequence of collaboration between aerobic glycolysis and reduced blood flow;^[24-26] therefore, despite of the acidity of tumors, most *in vitro* assays of tumor cell functions are routinely performed at neutral to alkaline medium pH.^[27] It has been found that increasing the pH of tumors causes a drastic reduction of *in vivo* cancer metastases.^[28] Therefore, alkaline pH is detrimental to the growth of cancer cells.

Table 1: Comparative analysis of effect of pH of medium versus AI extract and medium pH on breast cancer cell survival

S. No	pH	Cells/ml		% Cytotoxicity (as function of pH*)	Live Cell no. in AI extract (1600 µg/ml) treated well versus control well as function of pH (cells/ml)		% Cytotoxicity
		Live	Dead		Control	Experimental	
1	6.2	7.86 × 10 ⁵ ± 22.12	0.76 × 10 ⁴ ± 10.21	0.96 ± 5.20	5.66 × 10 ⁵ ± 18.25	2.43 × 10 ⁴ ± 12.52	95.7 ± 8.52
2	6.5	2.71 × 10 ⁵ ± 18.25	1.38 × 10 ⁴ ± 8.23	4.85 ± 2.15	4.56 × 10 ⁵ ± 16.14	2.95 × 10 ⁴ ± 11.10	93.5 ± 5.21
3	6.8	2.6 × 10 ⁵ ± 15.20	1.48 × 10 ⁴ ± 9.21	5.39 ± 7.21	2.56 × 10 ⁵ ± 11.23	2.14 × 10 ⁴ ± 10.25	91.6 ± 3.52
4	7.1	2.13 × 10 ⁵ ± 17.08	2.34 × 10 ⁴ ± 11.23	9.88 ± 6.32	2.0 × 10 ⁵ ± 12.54	2.9 × 10 ⁴ ± 16.24	85.5 ± 4.25
5	7.4	2.19 × 10 ⁵ ± 18.15	2.76 × 10 ⁴ ± 10.05	11.2 ± 5.24	1.87 × 10 ⁵ ± 15.23	3.76 × 10 ⁴ ± 14.23	79.9 ± 2.89

Table 2: Dose dependent effect of ethanolic extract of AI leaves on MDA cells as function of pH

AI Extract Dose (µg/ml)		Live Cell No. (cells/ml)		
		Control	Experimental	% Cytotoxicity
pH 6.2	200	$6.93 \times 10^5 \pm 12.34$	$1.38 \times 10^5 \pm 18.26$	80.1 ± 6.52
	400	$1.03 \times 10^6 \pm 11.22$	$1.36 \times 10^5 \pm 17.25$	86.8 ± 8.29
	800	$1.42 \times 10^6 \pm 15.22$	$9.34 \times 10^4 \pm 20.23$	93.4 ± 7.25
	1600	$5.66 \times 10^5 \pm 13.22$	$2.43 \times 10^4 \pm 18.25$	95.7 ± 8.26
pH 6.5	200	$3.63 \times 10^5 \pm 9.01$	$7.48 \times 10^4 \pm 12.23$	79.4 ± 8.26
	400	$1.64 \times 10^5 \pm 5.36$	$3.05 \times 10^4 \pm 14.25$	81.4 ± 3.65
	800	$3.89 \times 10^5 \pm 11.23$	$5.24 \times 10^4 \pm 16.25$	86.5 ± 5.78
	1600	$4.56 \times 10^5 \pm 9.26$	$2.95 \times 10^4 \pm 12.23$	93.5 ± 6.23
pH 6.8	200	$2.38 \times 10^5 \pm 5.02$	$9.48 \times 10^4 \pm 11.23$	60.2 ± 5.26
	400	$2.50 \times 10^5 \pm 8.06$	$7.25 \times 10^4 \pm 9.23$	71.0 ± 2.15
	800	$2.40 \times 10^5 \pm 2.28$	$4.20 \times 10^4 \pm 8.25$	82.5 ± 8.25
	1600	$2.56 \times 10^5 \pm 7.25$	$2.14 \times 10^4 \pm 13.25$	91.6 ± 5.69
pH 7.1	200	$1.78 \times 10^5 \pm 18.25$	$8.88 \times 10^4 \pm 20.13$	50.1 ± 4.88
	400	$1.89 \times 10^5 \pm 15.25$	$6.32 \times 10^4 \pm 15.26$	66.6 ± 5.23
	800	$1.93 \times 10^5 \pm 14.27$	$4.35 \times 10^4 \pm 11.20$	77.5 ± 3.29
	1600	$2.0 \times 10^5 \pm 23.51$	$2.90 \times 10^4 \pm 14.25$	85.5 ± 6.25
pH 7.4	200	$2.25 \times 10^5 \pm 12.25$	$7.32 \times 10^4 \pm 10.23$	67.5 ± 7.85
	400	$1.03 \times 10^5 \pm 9.26$	$2.67 \times 10^5 \pm 11.96$	74.1 ± 6.25
	800	$9.47 \times 10^5 \pm 10.28$	$2.25 \times 10^5 \pm 12.36$	76.2 ± 7.26
	1600	$1.87 \times 10^5 \pm 10.19$	$3.76 \times 10^4 \pm 14.26$	79.9 ± 8.29

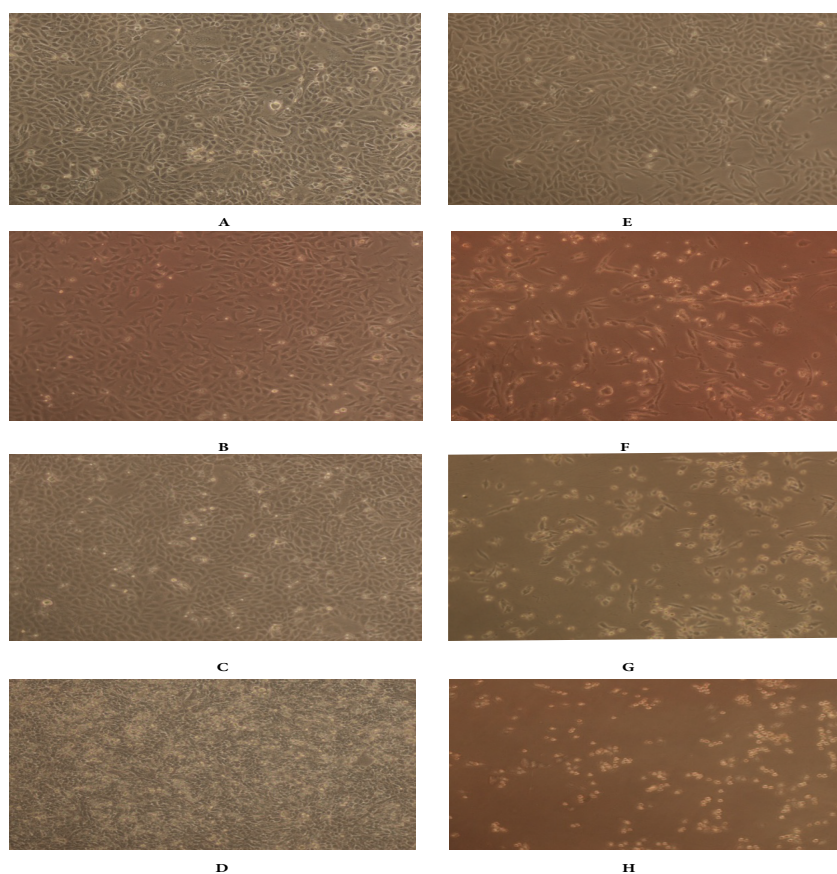


Figure 1: (A-D) Controls showing untreated MDA human breast cancer cells in presence of 1% DMSO at pH 6.2; (E-H) Dose dependent effect of AI ethanolic extract on MDA cells at 200, 400, 800, 1600 µg/ml respectively after 48 h (Magnification 10X)

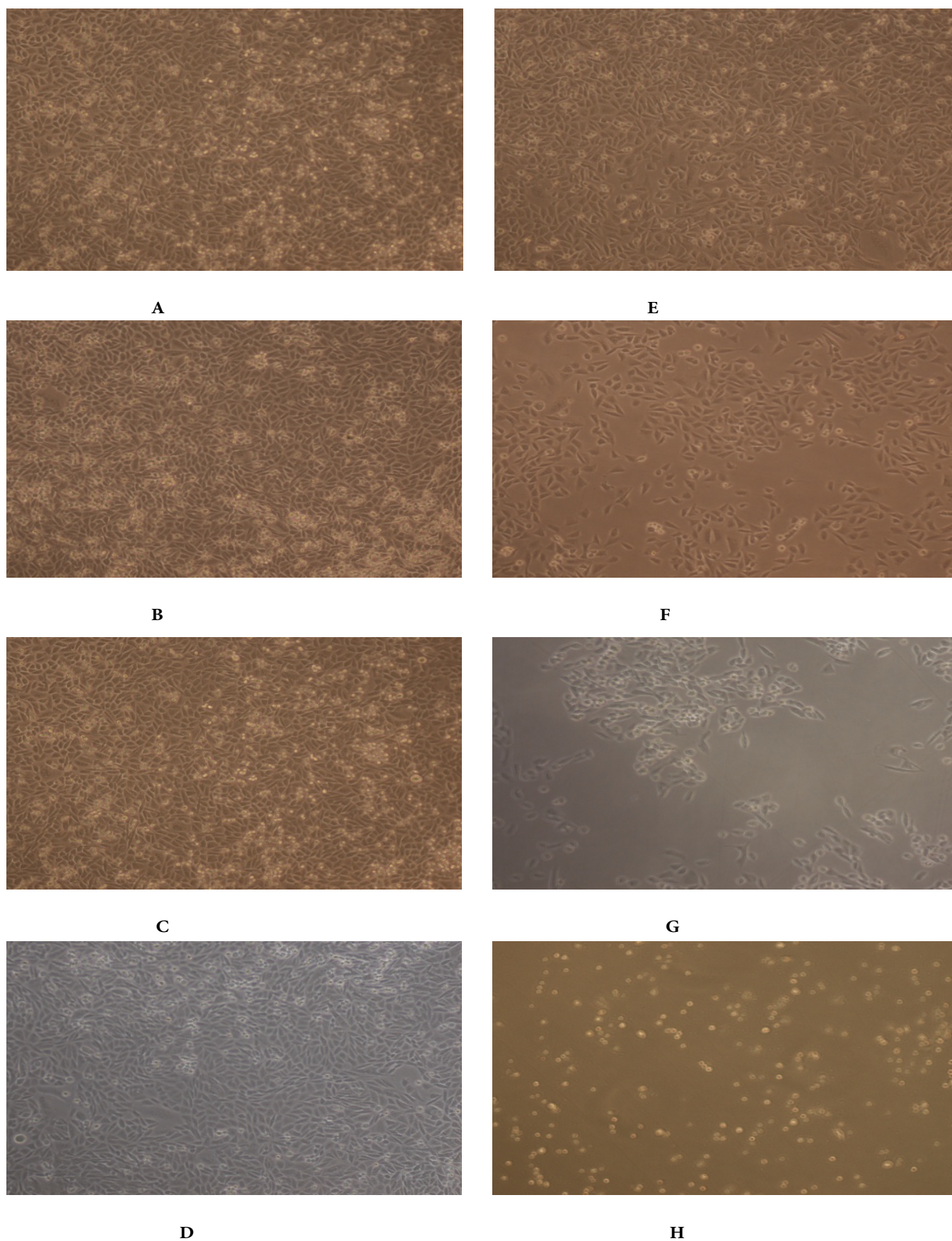


Figure 2: (A-D) Controls showing untreated MDA human breast cancer cells in presence of 1% DMSO at pH 6.5; (E-H) Dose dependent effect of AI ethanolic extract on MDA cells at 200, 400, 800, 1600 µg/ml respectively after 48 h (Magnification 10X)

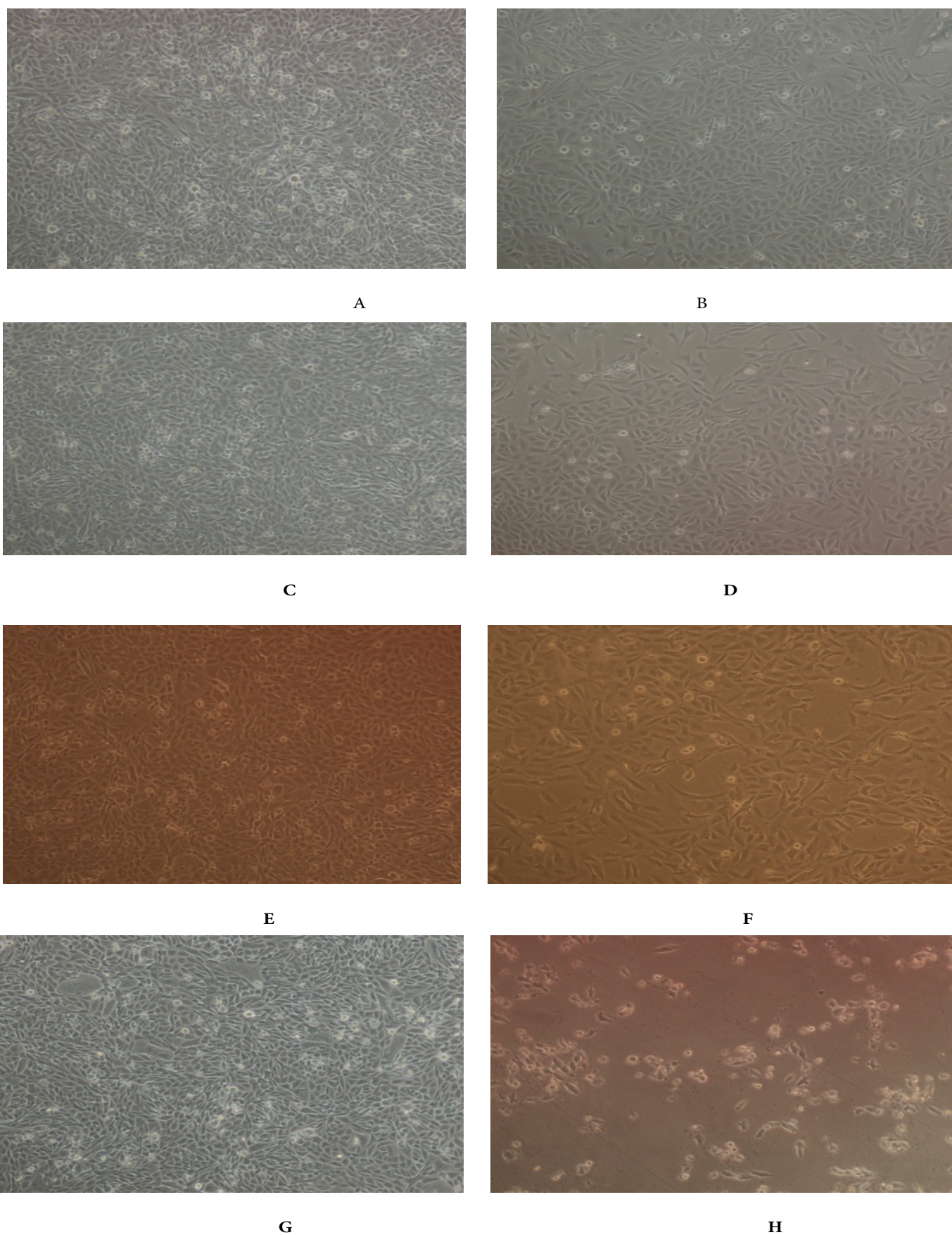


Figure 3: (A-D) Controls showing untreated MDA human breast cancer cells in presence of 1% DMSO at pH 6.8; (E-H) Dose dependent effect of AI ethanolic extract on MDA cells at 200, 400, 800, 1600 µg/ml respectively after 48 h (Magnification 10X)

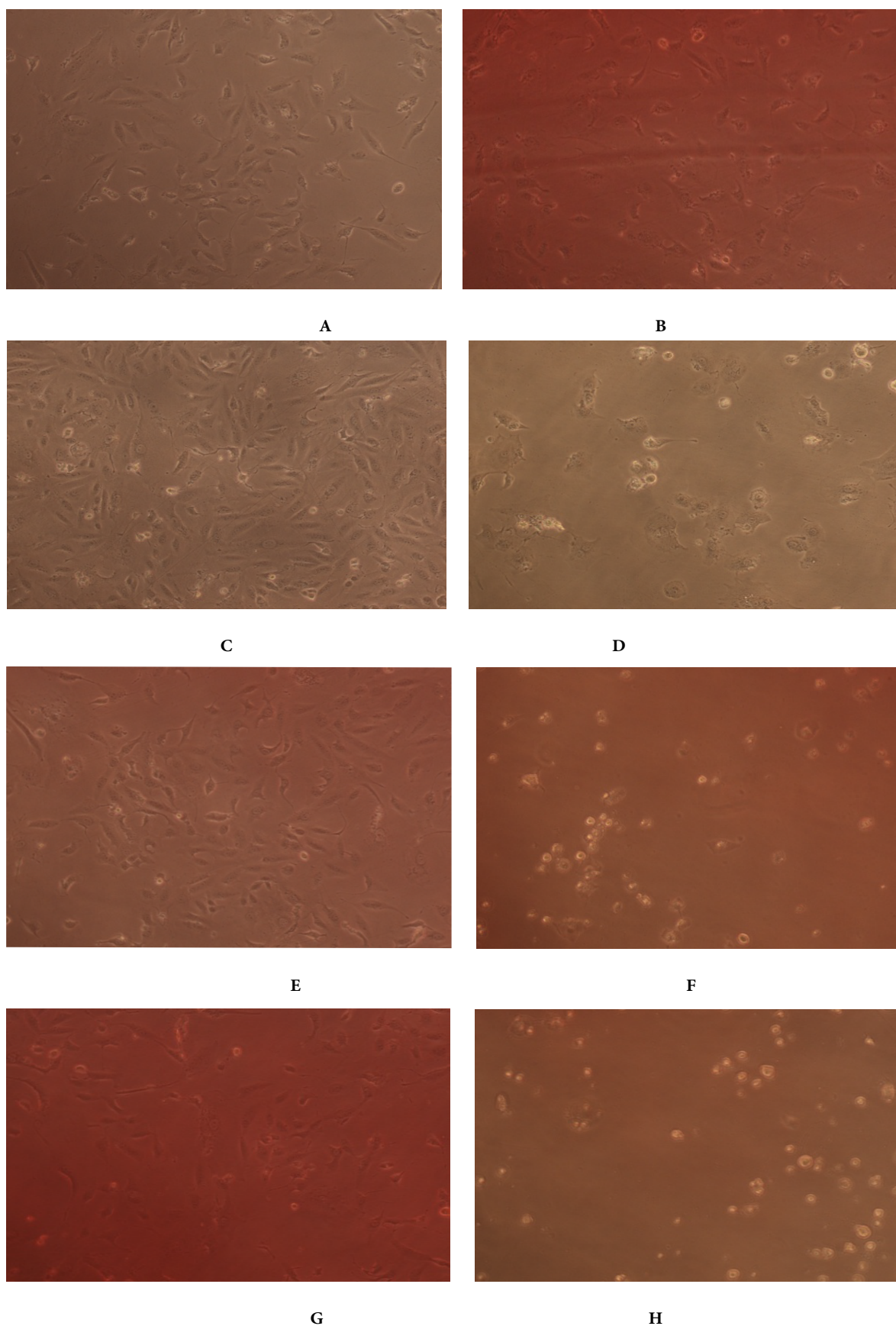


Figure 4: (A-D) Controls showing untreated MDA human breast cancer cells in presence of 1% DMSO at pH 7.1; (E-H) Dose dependent effect of AI ethanolic extract on MDA cells at 200, 400, 800, 1600 µg/ml respectively after 48 h (Magnification 10X)

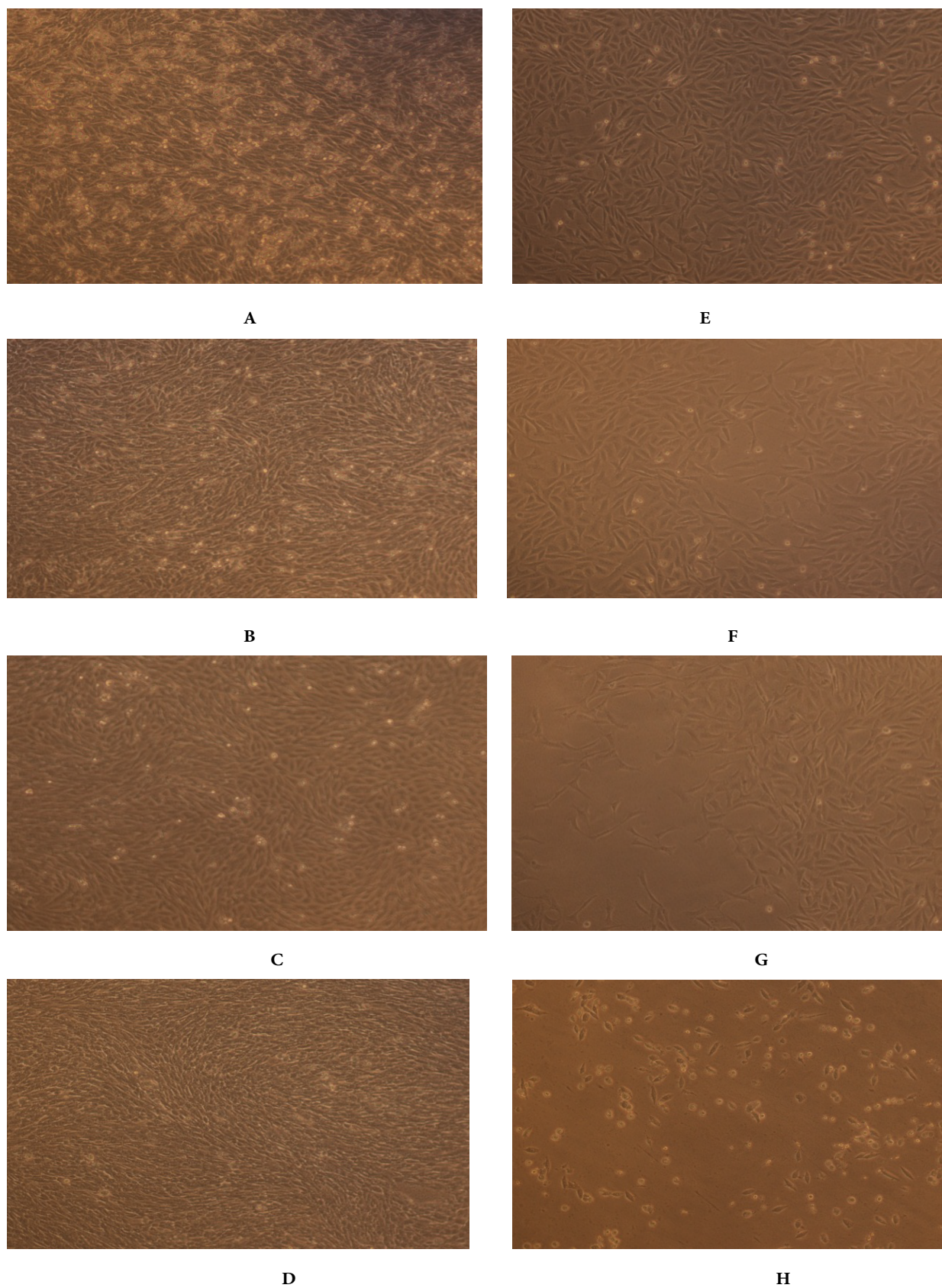


Figure 5: (A-D) Controls showing untreated MDA human breast cancer cells in presence of 1% DMSO at pH 7.4; (E-H) Dose dependent effect of AI ethanolic extract on MDA cells at 200, 400, 800, 1600 µg/ml respectively after 48 h (Magnification 10X)

Obokata *et al.* had recently claimed that low pH can induce stress in mammalian somatic cells and thereby trigger their reprogramming resulting in generation of pluripotent stem cells.^[28] However, the study was later found to be faulty as attempts to replicate the experiments failed^[29,30] and the papers were retracted and the conclusions were published later.^[31,32]

It is still a matter of great controversy as to whether cancer cell growth causes acidity (which promotes further cancer cell proliferation) or whether low pH is responsible for cancer cell proliferation. In the presence study, the combined effect of AI extract and pH was evaluated. It was found that AI caused significant reduction in breast cancer cell growth (95.7%) at pH 6.2. Ethanolic extract of AI performed better in an acidic medium probably because the phytoconstituents in AI extract had better solubility in acidic medium. Also, the acidic medium somehow facilitated the uptake of AI extract by the cancer cells. This observation needs further investigation and would be studied in future along with isolation and characterization of bioactive components in AI ethanolic extract.

CONCLUSION

The present study evaluated the cytotoxic activity of ethanolic extract of AI. In future, an attempt would be made to unravel and elucidate the molecular mechanism that facilitates uptake of drug (AI extract) by cancer cells at low pH.

Disclosures

None.

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.

Competing interests

The authors declare no conflict of interest.

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