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The Comparative Cytotoxic Investigation of Balanites Aegyptiaca (l.) Del Against Human Breast Cancer Cell Line (mcf-7) and Normal Cell Line (hbl-100) Through Apoptotic Pathway by Using Flow Cytometry Analysis

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ABSTRACT
The present investigation deals with cytotoxic investigation of Balanites aegyptiaca (L.) Del. Leaves by using MTT assay technique. Different extracts have been assessed against Human breast cancer cell line (MCF-7) and Normal cell line (HBL-100). The ethanolic extract shows potent cytotoxity against MCF-7 cell line. The efficacy of ethanolic extract against normal cell line have been confirmed through high IC50 and low percent inhibition. An apoptosis study was conducted on potent ethanolic extract against the MCF-7 cell line by using flow cytometry. Ethanolic extract showed late apoptosis & confirmed cell cycle arrest at the Sub G_0 phase. So ethanolic extract demonstrated potential to evacuate apoptosis on Human breast cancer cell line (MCF-7) and confirmed anticancer activity.

1. INTRODUCTION:

One of the most serious disorders is cancer, which is triggered proliferation of cells that destroy surrounding tissues. According to GLOBOCAN (Global Cancer) 2012 and the International Agency for Research on Cancer online database, it is the leading cause of morbidity and mortality worldwide, accounting for 8.2 million cancer-related deaths and 14.1 million new cases. Over the next 20 years, there is an expected 70% increase in cases. Radiation, chemotherapy, and surgery are among the clinical treatments that have been only partially successful; this suggests an urgent need for other approaches[1]. Due to the existence of secondary metabolites with therapeutic potential such as alkaloids, flavonoids, tannins, and phenolic, which are anticipated to be crucial in the treatment of cancer, medicinal plants have been utilized for ages to cure human ailments. Studies conducted both in vitro and in vivo have demonstrated the efficacious role of phenolic in the prevention or inhibition of disorders such as oxidative damage to DNA. Flavonoids can act by blocking the function of the cytoplasmic membrane, DNA gyrase and beta-hydroxyacyl-acyl carrier protein dehydratase. Balanites aegyptiaca (L.) Del plant's leaves show valuable presence of these active chemical constituents so it forms a strong basis as an alternative way for the treatment of cancer[2].

2. MATERIAL & METHOD:

2.1 In Vitro Anticancer Activity (MTT Assay method):

2.1 Experimental Protocol:

2.1.1 Collection of Plant Material:

Fresh Leaves and dried ripe fruits of *Balanites aegyptiaca (L.) Delile* was collected in the month of March-April from the rural region of Bhilavadi village. During this period, there was greatest prosperity occurred. Respective plant material was authenticated from the Botanical Survey of India, Pune.

2.1.2 Sample preparation[3]

The various extracts obtained after Successful soxhlat extraction of fresh leaves from *Balanites aegyptiaca (L.) Delile* plant. These extracts were subjected to further MTT assay. Respective extract

Journal of Molecular Science
Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 552-556
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samples were dissolved with 20 mg/ 10 ml PBS. These were vortexed for 30 minutes. 200 µl of DMSO (sigma, St. Louis, USA) was added to all the extract samples. An experimental procedure was carried out with these prepared extracts.

2.1.3 Cell lines and culture medium[4]:
MCF-7(Breast Cancer), HBL (Normal Cell Line) were purchased from NCCS, Pune, and cultures in Dulbecco's modified Eagle medium (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma, St. Louis, USA). The cells were then incubated in a humidified atmosphere at 5% CO_2 at 37^0 C. All the cell lines used in the study were of passage numbers between 3 to 7.

2.2 Experimental procedure (MTT Assay)[3,4,5]

1. Cells were incubated at a concentration of 1 × 10^4cells/ml in culture medium for 24 h at 37°C and 5% CO_2.
2. Cells were seeded at a concentration (70µl) 10^4cells/well in 100 µl culture medium and 100µl sample of LM series in (10,20,40,80,160µg/ml) into microplates respectively (tissue culture grade, and 96 wells).
3. Control wells were incubated with DMSO (0.2% in PBS) and the cell line. All samples were incubated in triplicate. Controls were maintained to determine the survival of control cells and the percentage of live cells after culture.
4. Cell cultures were incubated for 24 h at 37°C and 5% CO_2 in a CO_2 incubator (Thermo Scientific BB150)
5. After incubation, the medium was completely removed, and 20 µl of MTT reagent (5mg/min PBS) was added.
6. After the addition of MTT, cells were incubated for 4 hrs. at 37° c in a CO_2 incubator.
7. Observed the wells for formazan crystal formation under a microscope. The yellowish MTT was reduced to dark-colored formazan by viable cells only.
8. After removing the medium completely. Added 200µlof DMSO (kept for 10 min) and incubated at 37^0C (wrapped with aluminum foil).
9. Triplicate samples were analyzed by measuring each sample's absorbance using a microplate reader (**Benesphera E21**) at 550 nm wavelength.
10. A 50% inhibition (IC50) was determined. The percentage cell viability was determined utilizing the following formula:

Percentage cell viability = A550 of treated cells / A550 of control cells × 100
A550: Mean absorbance at 550nm
Growth inhibitory rate = $[1-(A_{550\ treated}/A_{550\ control})]$ ×100 %.

2.3 Apoptosis by Flow cytometer Protocol[6]-
The cells were seeded in a 6-well flat bottom microplate and maintained at 37^0C in a CO_2 incubator for overnight. The IC_{50} concentrations of each sample were treated at 24 hrs. After the incubation, cells were washed with PBS twice. Centrifuge for 5 minutes at 500 x g at 4°C. Discard the supernatant, and resuspend the cell pellets in ice-cold 1X Binding Buffer to 1 x 10^6 per mL. Keep tubes on ice. Then add 5 µl of AbFlour 488 Annexin V and 2 µL PI and Mix gently. Keep tubes on ice and incubate for 15 minutes in the dark. Add 400 µL of ice-cold 1X binding buffer and mix gently. Analyzed cell preparations within 30 minutes by flow cytometry. The analysis was done using FlowJo X 10.0.7 software.

3. RESULT & DISCUSSION:

3.1 In-vitro anticancer activity of Leaves from *Balanites aegyptiaca (L.) Delile*[6,8,9]
The various extracts available from *Balanites aegyptiaca's* leaves were tested against the Breast cancer cell line (MCF-7). For this purpose, the MTT assay technique was preferred. Concerned findings from this assay suggested that the ethanolic extract of leaves was more cytotoxic against both these cell lines in comparison to other extracts. 5-fluorouracil was used as the reference standard. Percent inhibition and IC50 values of extracts against MCF-7 were expressed in the following (Table no.2&fig.no.3). LD50 value was selected from reference review[8]. These findings also suggested that an increase in % inhibition or cell viability occurred in dose dose-dependent manner. (Table no.1 & fig.no.1,2)

Table no.1 Effects of compound against MCF-7 Cell line (Breast cancer cell line) by MTT Assay

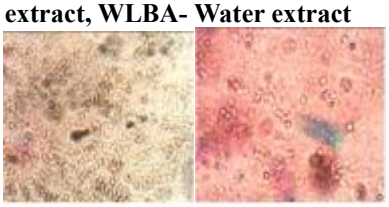
Sr. no	Sample	Concentration(µg/ml)	%Inhibition	IC50 (µg/ml)
1	**Control**	-	0.98	-
2	**Std. 5 FU**	10	28.32±0.48	91.15
		20	31.33±0.84	
		40	41.53±0.50	
		80	54.24±0.51	
		160	69.53±0.43	
3	**CLBA**	10	10.43±0.59	140.15
		20	14.96±2.32	
		40	29.61±0.99	
		80	42.78±0.76	
		160	50.47±2.31	
4	**ELBA**	10	27.50±0.58	92.08
		20	32.66±1.07	
		40	46.04±0.69	
		80	56.16±1.13	
		160	68.27±0.33	
5	**EABA**	10	27.82±0.70	115.38
		20	28.45±0.30	
		40	40.67±1.42	
		80	49.10±0.62	

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 552-556
DoI-10.004687/1000-9035.2025.075

		160	55.11±1.51	
6	**HLBA**	10	32.77±0.66	105.92
		20	33.83±1.04	
		40	42.99±0.57	
		80	47.63±0.77	
		160	51.53±0.40	
7	**WLBA**	10	0.21±0.78	>160
		20	1.47±1.05	
		40	3.79±1.32	
		80	6.32±0.87	
		160	9.69±1.04	

Data represented as replicates of 3 readings (± standard error of the mean)

CLBA- Chloroform extract, ELBA- ethanol extract, EABA- Ethyl acetate extract, HLBA- n-hexane extract, WLBA- Water extract



MCF- control **MCF-Treatment**

Fig no.1 % inhibition of MCF-7 by MTT assay

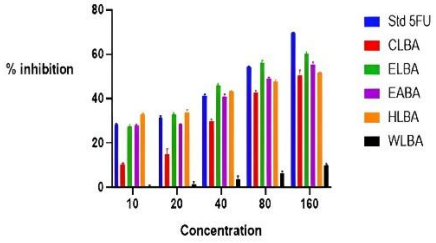


Figure no.2 Graph of Concentration Vs % inhibition for MCF-7 cell line

Table No.2 IC 50 values of extracts (MCF-7)

Sr. No	Extract	IC50
1	WLBA	160
2	CLBA	140
3	EABA	115.38
4	HLBA	105.92
5	ELBA	92.08
6	Std. 5 FU	91.15

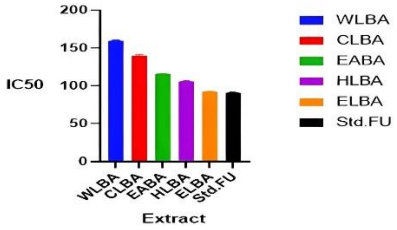


Figure no.3 Graph of Extract Vs % IC50 (MCF-7)

3.2 Normal Breast epithelial cell line (HBL-100)[10]

The same extracts from leaves of Balanites aegyptiaca were tested against a Normal Breast epithelial cell line (HBL-100) by using the same MTT assay procedure as previously used. Concern investigation regarding % inhibition and IC50 values were shown in below following **Table no.3.**

Table no.3 Effects of compound against HBL-100 Cell line by MTT Assay

Sr. no	Sample	Concentration(µg/ml)	%Inhibition	IC50 (µg/ml)
1	**Control**	-	-	-
3	**CLBA**	10	8.54±0.40	159.13
		20	10.22±0.66	
		40	12.11±0.87	
		80	20.30±0.71	
		160	21.47±0.63	
4	**ELBA**	10	14.32±0.55	178.27
		20	13.20±1.23	
		40	16.18±1.25	
		80	17.30±1.18	
		160	18.02±0.33	
5	**EABA**	10	14.54±1.25	160.28
		20	15.17±1.33	
		40	16.71±1.46	
		80	17.25±1.89	
		160	18.24±1.34	
6	**HLBA**	10	19.45±1.28	157.66
		20	20.23±1.54	
		40	27.32±1.29	
		80	24.25±1.26	
		160	26.13±0.89	
7	**WLBA**	10	8.22±1.56	174.56
		20	7.12±1.22	
		40	4.55±1.87	
		80	5.22±1.56	
		160	8.56±0.56	

Data represented as replicates of 3 readings (± standard error of mean)

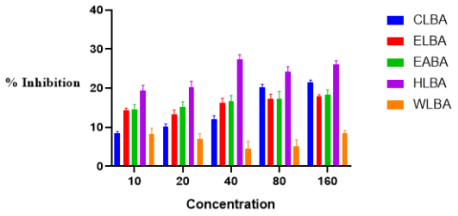


figure no.4 Graph of Concentration Vs % inhibition for HBL-100 Normal cell line

Table No.4 IC 50 values of extracts (HBL-100)

Sr. No	Extract	IC50
1	WLBA	160
2	CLBA	159.13
3	EABA	160.28
4	HLBA	157.66
5	ELBA	178.27

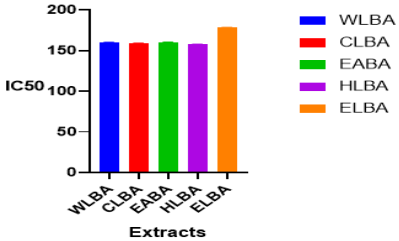


Figure no.5 Graph of Extract Vs % IC50 (HBL-100)

Journal of Molecular Science
Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 552-556
DoI-10.004687/1000-9035.2025.075

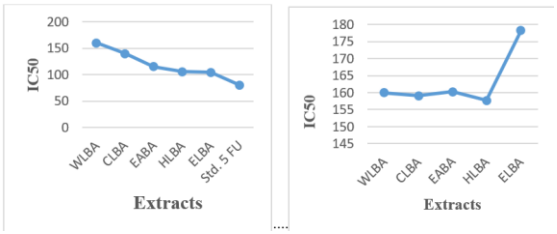


Figure no.6 Comparative IC50 values for MCF-7 & HBL-100

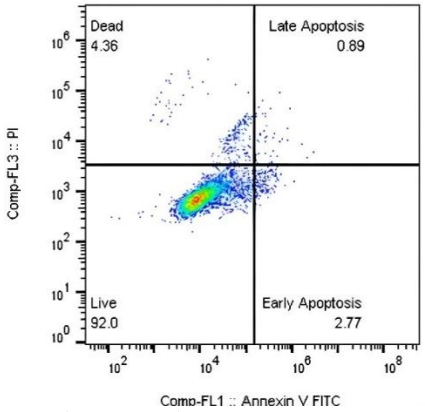
These findings from Table no.16 suggested that no extract showed considerable percent inhibition against HBL- 100 which was treated as a normal Breast epithelial cell line in comparison to MCF-7 cell line treatment. So, no cytotoxicity was considered against it.IC-50 values for these test extracts against HBL-100 were also high compared to the breast cancer cell line **(Table no.4& fig.no. 5,6),** so it was assumed to be safe for human beings.

3.3 Apoptosis Study[7,11]

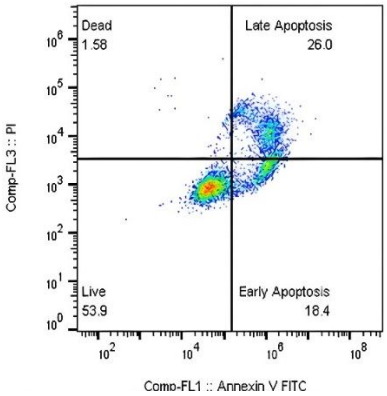
Previous findings from the MTT assay suggested that Ethanolic extract (ELBA) of Balanites aegyptiaca's leaves showed considerable cytotoxicity against the MCF-7 cell line.so, only these findings were considered for apoptosis study. The present study was conducted by using Annexin V & Propidium iodide (PI) treatment. The following findings from **Table No. 5** clearly express the outcome of this study.

Table no.5 Assumptions from Apoptotic Study

Cell code	Annexin V	Propidium iodide
Apoptotic	+	-
Dead	+	+
Viable/Live	-	-



a. Control (untreated)



b. ELBA (Treated)

Figure no.7 Flow cytometric analysis

(Q1- Dead cell, Q2- Late apoptosis, Q3- Early apoptosis, Q4- Live/viable cell):

The figure no.7 promptly revealed results from Annexin V- FITC analysis. These findings were created through a comparison between the Control (untreated) sample (A-MCF-7 Cell line) & treated Test sample (B-MCF-7 treated with ELBA-ethanolic extract of Balanites aegyptiaca's leaves). It was expressed that cells are distributed between four quadrants (Q1, Q2, Q3, Q4). The test sample allowed the formation of early and late apoptosis. Less proportion of live cells was seen in the test sample which decreased from 92% to 53.9%. The control sample showed no considerable cell distribution within Q1, Q2 & Q3 while it was considerably increased in the test sample during Annexin V- FITC treatment. The proportion of late apoptosis (Q2) was considerably increased (from 0.89% to 26%) in treated cells. It was time-dependent. **Fig no.7** clearly showed there was no considerable change in dead cell proportion (Q1) during flow cytometry analysis. so, these findings from Flow cytometry analysis effectively suggested that Ethanolic extract of Balanites aegyptiaca's leaves (ELBA) allowed the creation of 26% late apoptosis in the MCF-7 cell line.

Table no.6 Observations from Annexin V- FITC analysis

Sr. No	Q4	Q3	Q2	Q1
Sample code	Live	Early apoptosis	Late apoptosis	Dead
Control(untreated)	92	2.77	0.89	4.36
ELBA (treated)	53.9	18.4	26	1.58

Therefore, the goal of the present research was to focused on the molecular mechanisms underlying the bioactive substances' ability to prevent apoptosis and the mechanism of cell death in MCF-7 cells used as in vitro models. Phospholipids in the plasma membrane are erratically dispersed throughout the inside and outside leaflets. Phosphatidylcholine and sphingomyelin are brought out by the lipid bilayer's

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 552-556
DoI-10.004687/1000-9035.2025.075

external leaflet, while phosphatidylserine is revealed by the inner leaflet. Apoptosis disrupts this asymmetry, exposing phosphatidylserine on the outside surface of the plasma membrane. When the cells are incubated with ELBA, a decrease in viability is seen during Annexin V- FITC analysis, suggesting that ELBA has a pattern of inhibiting MCF-7 cell growth through an apparent build-up of cells in the Sub-G0 phase. Our results demonstrated that ELBA administration triggered Sub-G0 phase cell cycle arrest and apoptotic cell death, which delayed the progression of MCF-7 cells through the G1, S, and G2/M phases of the cell cycle.

4. DISCUSSION:

In the present study, successive extracts from leaves of *Balanites aegyptiaca (L.) Delile* plants were subjected to in-vitro anticancer activity against the MCF-7 cell line (Breast cancer). For that purpose, the MTT assay method was preferred. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced by mitochondrial dehydrogenases inside living cells. This reduction forms purple formazan crystals. The degree of light absorption by the formazan product correlates with cell viability or percent inhibition. The ethanolic extract of *Balanites aegyptiaca* leaves showed potent activity against the MCF-7 cell line along with a significant IC50 value (Table no.1). These extracts were also tested against Brest normal cell line (HBL-100).no extracts showed cytotoxicity against it so it will be considered as safe use for human being. An apoptosis study was conducted on potent ethanolic extract against the MCF-7 cell line by using flow cytometry. Ethanolic extract showed late apoptosis & confirmed cell cycle arrest at the Sub G_0 phase.

5. CONCLUSION

The various extracts from leaf sample were subjected towards in-vitro anticancer activity by using MTT assay method. For that purpose, MCF (Breast cancer cell line) was preferred. These extracts (especially ethanolic extract) were shown promising results against MCF-7 cell line. These extracts were also tested against HEP-100 cell line (Normal breast cell line) & confirms safety concern towards human being through low percent inhibition & high IC50 value.

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