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In Vitro Cytotoxicity Studies of Indian Herb Tribulus Terrestris on Human Breast Cancer Cells

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Abstract: *Tribulus terrestris (family Zygophyllaceae), commonly known as Gokharu or Punture wine has been used since long in Indian medicine for the treatment of various kind of diseases. The main active compounds of the plant are the steroidal Saponins. The present study aims to analyze the cytotoxic effect of methanolic and Saponin extracts ofTribulus terrestrison human breast cancer line MCF-7. The cytotoxic effect was established by MTT, Sulforhodamine B and LDH cell viability assays. Using MTT assay, an extensive cytotoxic analysis was done for 15 extracts (prepared by different methods in different solvents) along with Saponin fractions of leaf and seed parts of the plant testing concentrations ranging from 6.25 µg/ml to 100 µg/ml, the extracts showing reasonable cytotoxicity were selected for further assays. The MTT assay results showed that 5 methanolic extracts and Saponin fractions have distinct cell cytotoxicity activity specifically showing higher activity at lower concentration 25, 12.5 and 6.25 µg/ml. IC_{50} values of leaf and seed Saponins was found to be 6.25 µg/ml and 12.5 µg/ml. Nine out of seventeen extracts showed cytotoxicity selected for subsequent assays. Results of Sulforhodamine B assay showed similar results as of MTT assay with lower concentrations showing higher activity. LDH assay results showed 23.81% and 11.13% cytotoxicity of seed and leaf Saponins at 100 µg/ml and 12.5 µg/ml concentration respectively. Methanolic extracts of leaf and seed in this case have shown cytotoxicity of 13.12% and 7.27% at 25 µg/ml concentration respectively.This study gives direction for future investigation concerning detailed assessment of pharmacological potential of Tribulus terrestris.*
Keywords: *Tribulus terrestris, Saponins, Cytotoxicity, Human Breast Cancer cells*

I. Introduction

Breast cancer is the commonest cancer in women worldwide with a widely variable incidence between countries and regions.In India, breast cancer is the second most common cancer (after cervical cancer) with an estimated 115,251 new diagnosis and the second most common cause of cancer-related deaths with 53,592 breast cancer deaths in 2008 (Ferlay*et al*.,2000). Due to the lack of effective drugs, cancer is fatal disease rating the top three cause of death. Many of the chemotherapeutic agents sold for the treatment of cancer is highly expensive, mutagenic, carcinogenic and teratogenic (Kumarappan*et al*., 2007). Therefore the quest for effective anti-cancer drug is a need of an hour. *Tribulus terrestris* is a well patronized medicinal herb by ayurvedic seers as well as by modern herbalists. It is commonly known as Gokharu or Puncture vine and belongs to the family Zygophyllaceae. Historically, *Tribulus terrestris* was used by the cultures of India and Greece as a rejuvenation tonic (Adaikan*et al*., 2000). The entire plant (and particularly the fruits) is used in an indigenous system of medicine for treating various ailments, such as urinary infections, eye infections, leucorrhoea, vitiligo, and impotence (Wang *et al*., 1997). The therapeutic properties of *Tribulus terrestris* have been attributed to the presence of active compounds like Saponins, Alkaloids, Flavonoids etc. (Anita *et al*., 2013).Saponins are glycosides with foaming characteristics. Among many of the diversified roles of Saponins, one of the much explored roles is as an anti-cancerous agent. Saponins have been reported to inhibit proliferation and induce apoptosis of tumor cells thus reducing their invasive activity. Triterpene Saponins have multidirectional effects on cancer cells (Koczurkiewicz*et al.*, 2015). The inhibitory effect of Saponins from *Tribulus terrestris* on Bcap-37 breast cancer cell line *in vitro* were also studied (Zhong Yao Cai., 2003). Another study showed that *Tribulus terrestris* aqueous extract blocks proliferation and induces apoptosis in human liver cancer cells through inhibition of Nf-kBsignaling and can be used as an anticancer drug for hepatocellular carcinoma patients (Kim *et al*., 2011). Recent study done by Angelova*et al*. (2013) reveals that Bulgarian herb *T.terrestris* has anticancer activity on human breast cancer cells. Present study aims to investigate the cytotoxic effects of methanolic and Saponin extracts of leaves and fruit of*Tribulus terrestris* on Human Breast Cancer cells MCF-7 bythree different assays MTT, Sulforhodamine B (SRB) and Lactose Dehydrogenase (LDH).

II. Materials And Methods

2.1 Plant Collection

*Tribulus terrrestris*L.commonly known as Gokharu was collected during its fruiting period(December-March) from Junagham, near Suvalli, Dist. Surat, Gujarat, Latitude - 21.141400 and Longitude-72.63525. The plant material was properly washed under tap water and finally rinsed with distilled water. The leaves and fruits

were dried separately in oven at 60^{o}C. After drying, the plant parts were grounded well into fine powder and stored in air tight container.

2.2 Preparation of Crude Extracts

2.2.1 Soxhlet Extraction

5 grams of dried leaf powder and 10 grams of dried seed powder were extracted in Soxhlet extractor using 150 ml of methanol& acetone (60-80^{o}C) until a clear sample was obtained (approx. 6 hrs, 8 cycles). At the end of extractions, the extracts were filtered & distilled to concentrate. The residues were dried under vacuum for 24 hrs and yield was recorded.

2.2.2 Preparation of Aqueous and Organic Extracts

Aqueous and methanolic crude extracts were prepared according to protocol described by Sanjay *et.al.*(2013). Five gram of each dried leaf and seed was macerated in 100 ml of methanol and distilled water for 72 hours, filtered through Whatman's filer paper no. 1, dried at room temperature and store at 4^{o}C in refrigerator.

2.2.3 Solvent Partition Extraction

The crude methanolic extract of *Tribulus terrestris* was subjected to bio-guided fractionation by solubilisation in water and sequential partition with hexane (5×400mL), chloroform (3×400mL), ethyl acetate (5×400mL), and 50% methanol (3×400mL) as indicated in Fig. 1. Each fraction thus obtained, including the final hydro-methanol fraction, was evaporated to dryness and resuspended inrespective solvents (Maryam *et.al.* 2012).

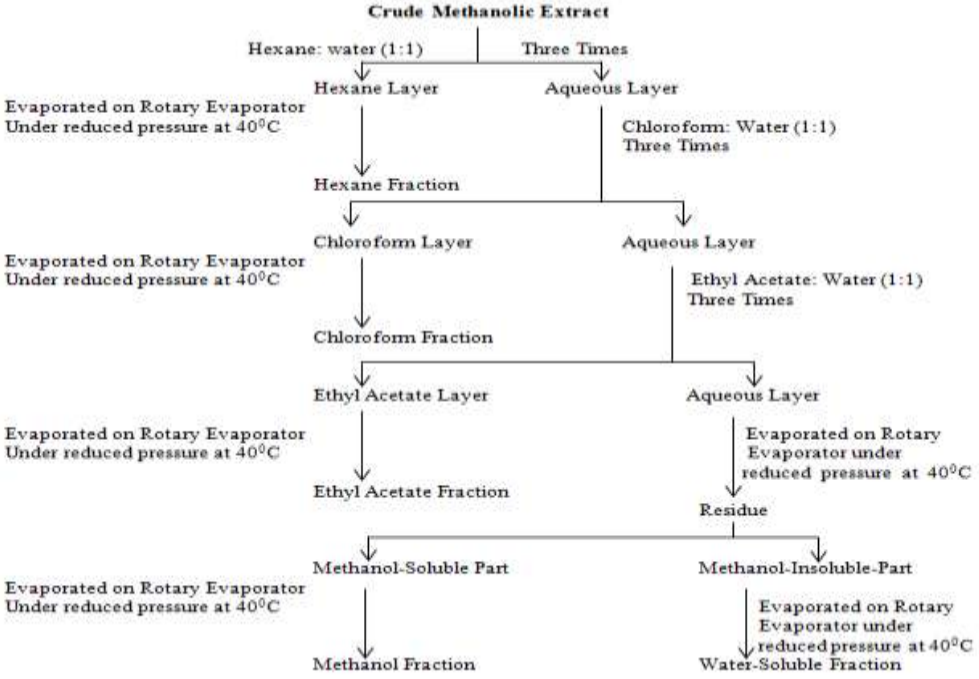


Figure 1: Chart Diagram of Solvent Partiton Method

2.2 Determination of Saponins

Saponin extraction was done according to method described by Obadoni and Ochuku with slight modifications (Obadoni and Ochuku, 2001). 5 grams of dried leaf and 10 grams of seed powder wereheated with continuous stirring at 55^{o}C in 100 ml and 50 ml of 20% aqueous ethanol respectively. The mixture was filtered and the residue re-extracted with 100 ml of 20% aqueous ethanol. The combined extracts were reduced to 60 ml over water bath at about 90^{o}C. The concentrates were defatted with 15 ml diethyl ether and finally extraction was carried out by adding 45 ml of n-butanol. The combined n-butanol extracts were washed twice with 11.5 ml of 5% aqueous NaCl solution. The remaining solution was evaporated to a constant weight in water bath and the Saponin content was calculated as percentage.

2.3 Cell Line Acquisition and Maintenance

Human Breast Cancer cell line MCF-7 was procured from National Centre for Cell Sciences (NCCS), Pune and cultured in Minimal Essential Media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), (100U) 20µg/ml Penicillin, and 100µg/ml Streptomycin at 37°C with an atmosphere of 5% CO_2.

2.4 Cytotoxicity Analysis

2.4.1 MTT Assay

The anticancer activity of different extracts on MCF-7, were determined by the MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay to assess the cytotoxicity according to the method described by Horiuchi*et al*., (1988).For MTT assay, cells were plated in 96-well plates at the density of $5X10^3$/100µl. After 24 hof incubation, cells were treated with the crude and Saponin extracts at concentrations of 6.25, 12.5, 25, 50 &100 µg/ml. Wells with serum-free medium were used as negative controls. After 24 h incubation at 37°C, 10 µl MTT reagent was added to each well and incubated for further 4 hin dark at 37°C. Then, 100 µl of Sorenson glycine buffer (0.1M glycine, 0.1M NaCl, pH 10.5 with 0.1N NaOH) was added to the wells to solubilize the formazan crystals. The absorbance was subsequently measured at 490 nm. The experiment was repeated 3 times and each concentration was tested in triplicates. The percentage viability of cells was calculated.

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Inhibition (%) = 100 - Cell Viability (%)

2.4.2 SRB Assay

The Sulforhodamine B (SRB) cell viability assay is a non-destructive colorimetric endpoint assay for determination of cell proliferation and cell cytotoxicity. The assay estimates cell number indirectly by staining total cellular protein with the SRB dye. MCF-7 cells were plated in 96-well plate at the cell density of $5X10^3$/100µl. After 24 hof incubation, concentrations of extracts showing highest cell cytotoxicity in MTT assay were selected for cell treatments.After 24 hrs, cell staining, colorimetric analysis was performed according to the manufacturer's protocol usingEzcount™ Sulforhodamine B cell assay kit (Himedia).

2.4.3 LDH Assay

The Lactose dehydrogenase (LDH) assay was carried to confirm the percentage cell cytotoxicity of the extracts on MCF-7 cells. In LDH assay, the plasma membrane enzyme LDH when released by lysed cells reacts with pyruvate which reacts with tetrazolium salt (INT) to form formazan. The amount of formazan released is proportional to the number of lysed cells. The cells were plated in 96-well plate at a cell density of $5X10^4$/100µl. After 24 h of incubation, same concentrations of extracts that were used for SRB assay were used for cell treatments. After 24 hrs, LDH assay was performed according to the manufacturer's protocol using Ezcount™ LDH cell assay kit (Himedia).

2.5 Statistical Analysis

Data were expressed as mean ± SD. Statistical comparisons of the results were made using one way analysis of variance (ANOVA). Differences between the means of control and treated cells were examined using Bonferroni t-test. P value <0.05 was considered significant.

III. Results

Seventeen extracts were prepared from leaves and seeds of *Tribulus terrestris*by utilizing three extraction methods Manual, Solvent Partition and Soxhlet with the use of different solvents. Table 1showed the results of the extracts formation alongwith their respective concentrations.

Table 1: Details of the extracts prepared from different parts of *Tribulus terrestris*

S. No	Extract Name	Concentration (mg/ml)
Leaf Extracts		
1.	Soxhlet with Methanol	155.4
2.	Acetone Soxhlet	52
3.	Methanol Manual	217
4.	Aqueous	160.6
5.	n-Hexane serial	27
6.	Chloroform serial	20
7.	Ethyl acetate serial	25
8.	Methanol serial	44.66
9.	Leaf Saponin	52.5
Seed Extracts		
10.	Methanol with Soxhlet	61.8
11.	Acetone with Soxhlet	104
12.	Aqueous	179.4
13.	n-hexane serial	50.66

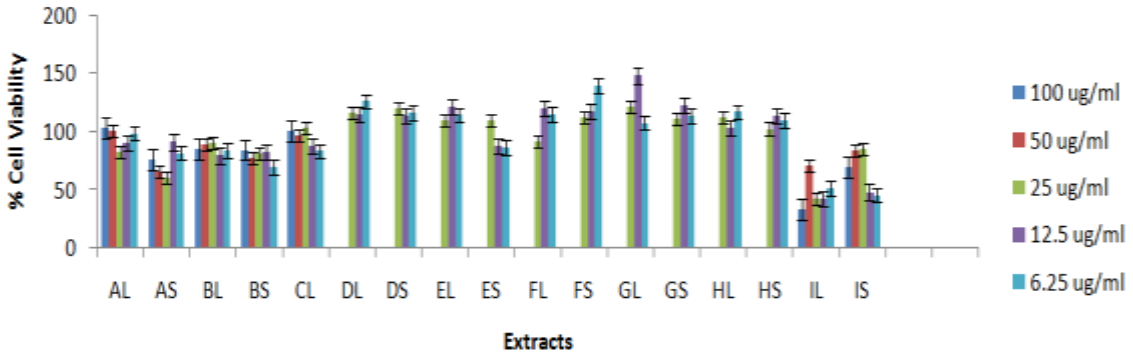
14.	Chloroform serial	22.5
15.	Ethylacetate serial	13.5
16.	Methanol serial	72.5
17.	Seed Saponin	32.5

3.1 MTT assay

MTT results revealed that 9 out of 17 tested extracts shows profound cytotoxicity including methanolic and Saponin extracts of leaf and seed prepared by different methods (Fig. 2). Chloroform, n-hexane, acetone, ethylacetate and aqueous extracts of leaf and seed didn't show any activity. The results also reflect that the inhibiting function of extract is not dose dependent. It was seen that seed methanolic extract was far more effective in reducing the cell viability as compared to leaf methanolic extract with lowest cell viability value of 60.53% ($P<0.05$) at 25 µg/ml concentration when extracted by soxhlet extraction while leaf methanolic extracts showed lowest cell viability of 67.64% at 6.25 µg/ml concentrations when extracted by solvent partition (serial exhaustion method). Comparing the methods of extraction, it can be concluded that Soxhlet extraction method was more effective in extracting the effective compounds as compared to other two methods. On the basis of these results we have selectedtwo leaf and seed extracts of 25 and 12.5 µg/ml each, for further studies.The data showed promising results in case of Saponins extracts. Leaf Saponin has been able to reduce cell viability up to 33.23% at 100 µg/ml concentration and up to 42.48% at 12.5 µg/ml concentration (with IC_{50} of 6.25µg/ml). Similarly, seed Saponin was able to reduce cell viability up to 48.25% and 45.52% at 12.5 µg/ml and 6.25 µg/ml concentrations respectively (with IC_{50} of 12.25µg/ml).

Table 2: Details of the extracts showing cytotoxic activity when tested by MTT assay

Sr.No	Extract	Concentration (µg/ml)				
		100	50	25	12.5	6.25
1	Soxhlet (Leaf)	--	--	√	√	√
2	Soxhlet(Seed)	√	√	√	√	√
3	Serial(Leaf)	√	√	√	√	√
4	Serial(Seed)	√	√	√	√	√
5	Manual(Leaf)	--	√	--	√	√
6	Acetone(Leaf)	--	--	--	--	--
7	Acetone(Seed)	--	--	--	--	--
8	Aqueous(Leaf)	--	--	--	--	--
9	Aqueous(Seed)	--	--	--	√	√
10	n-Hexane(leaf)	--	--	√	--	--
11	n-Hexane(Seed)	--	--	--	--	--
12	Chloroform(Leaf)	--	--	--	--	--
13	Chloroform(Seed)	--	--	--	--	--
14	Ethyl Acetate(Leaf)	--	--	--	--	--
15	Ethyl Acetate(Seed)	--	--	--	--	--
16	Saponin(Leaf)	√	√	√	√	√
17	Saponin(Seed)	√	√	√	√	√
•	**√ = showing cytotoxic activity -- = Showing no cytotoxic activity**					



Abbreviation*- AL-leaf methanol soxhlet, AS- seed methanol soxhlet, BL- leaf methanol serial, BS-seed methanol serial, CL-Leaf methanol manual, DL-leaf acetone, DS-seed acetone, EL-Leaf aqueous, ES- seed aqueous, FL-leaf n-hexane, FS-seed n-hexane, GL-leaf chloroform, GS-seed chloroform, HL-leaf ethyl acetate, HS-seed ethyl acetate, IL-leaf Saponin, IS-seed Saponin*

Figure 2:MTT assay of MCF-7 cell lines treated for 24 hour with increasing range of concentrations of different extracts of *Tribulus terrestris*

3.2 SRB and LDH assay

SRB assay results shows that Seed methanolic extract was far more effective than Leaf methanolic extract by reducing cell viability up to 76.31% at 25 µg/ml concentration when extracted by Soxhlet method and 74.82% of cell viability reduction was noted at 6.25 µg/ml when extracted by solvent partition method (Fig. 3, Table 3). Leaf methanolic extract was able to decrease cell proliferation by highest 73.79% at 6.25 µg/ml when extracted by Soxhlet method. Moreover, seed Saponin fraction have shown highest activity at 25 µg/ml concentrations by reducing cell viability upto 69.33% and leaf Saponin fraction similarly reduced cell viability up-to 72.19% at 25 µg/ml concentration. The results of LDH assay further supports the cytotoxic activity of *Tribulus terrestris.* Similar to the MTT and SRB assay results, the Saponin fractions of seed was more effective in reducing cell viability with the highest cytotoxicity of 23.81% at 100 µg/ml concentration (Fig. 3). Leaf Saponin extract have shown highest cytotoxicity of 11.13% at 12.5 µg/ml concentration. Methanolic extracts of leaf and seed have shown cytotoxicity of 13.12% and 7.27% at 25 µg/ml concentration respectively when calculated by LDH assay.Thus, the data further confirmed that the Saponins are the main active cytotoxic compounds present in *Tribulus terrestris.* This led us to carry out on-going research of purifying and identifying the active Saponins of *Tribulus terrestris.* Moreover, the data also points towards that seeds of *Tribulus terrestris* are rich in cytotoxic compounds (specifically Saponins) as compared to its leaves.

Table 3: Details of the extracts showing cytotoxic activity when tested by SRB and LDH assay

Sr.No	Extract	Concentration	SRB Assay		LDH assay
			%Cell Viability	% Inhibition	% cell Cytotoxicity
1.	Leaf Methanol soxhlet	25	86.15	13.85	13.12
		12.5	82.60	17.40	7.49
2.	Seed Methanol Soxhlet	25	76.31	23.69	7.27
		12.5	77.11	22.89	3.74
3.	Leaf Methanol Serial	12.5	74.02	25.93	-2.31
		6.25	73.79	26.21	1.10
4.	Seed Methanol Serial	50	73.34	26.66	1.43
		6.25	74.82	25.18	2.75
5.	Leaf Saponin	100	90.50	9.50	-6.17
		50	77.80	22.20	-8.59
		25	72.19	27.81	-1.21
		12.5	73.91	26.09	11.13
		6.25	77.45	22.55	10.03
6.	Seed Saponin	100	78.37	21.63	23.81
		50	70.36	29.64	15.65
		25	69.33	30.67	13.56
		12.5	83.29	16.71	6.83
		6.25	76.08	23.92	14.44
7.	Seed Aqueous	12.5	70.25	29.75	7.05
		6.25	75.51	24.49	14.33
8.	Leaf n-Hexane	25	70.93	29.07	11.68
		6.25	68.87	31.13	-12.34

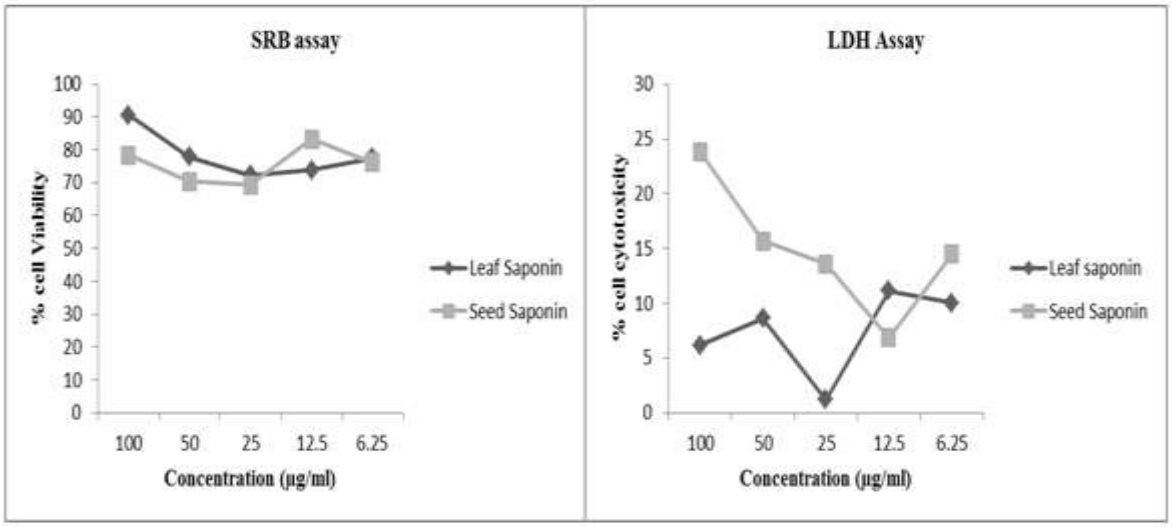


Figure 3: SRB and LDH assay of MCF-7 cell line treated for 24h with Saponin extracts of *Tribulus terrestris*

IV. Discussion

Plant based drugs have been used since ages to treat various ailments. Recently, plant-based products are in search which can be used as a potential source of anti-cancer agents. According to one study, out of 25000 species present on earth, 1000 have reported to have anti-cancer potential (Mukherjee *et al*., 2001). Due to limitation and flaws of chemotherapeutic agents commonly used to treat cancer, the need to search new anti-cancer agents which are plant based have been increased.*Tribulus terrestris*formulation has been used to treat various ailments. Recently, anti-proliferative effect of this plant against breast cancer cells was reported. Therefore, the present study aims to study the cytotoxicity of this plant on human breast cancer cells MCF-7. The results of the performed MTT analysis showed that the extract of *Tribulus terrestris* does not have a dose dependent inhibitory effect on viability of human breast cancer cells. In contrast, the lower concentration (12.5 µg/ml and 6.25 µg/ml) has stronger inhibitory effect as compared to higher doses (100 µg/ml and 50 µg/ml). The reason for this high variability is probably due to the different chemical nature and composition of active Saponins in *T. terrestris* from different geographical areas, and also to differences in the extent and mechanism of their action. The Indian herb is rich in TribulosinSaponins while Bulgarian herb is rich in FurostanolSaponins, which are smaller in amounts or missing in the plants from Asian region (Dinchev*et al*., 2008). Of importance for the differences in cytotoxic activity is also the type of treated tumor cells. In this connection Hu & Yao (2001) found that from the 60 analysed cell lines most sensitive to methyl protogracillin are the cell lines from leukaemia, Colon cancer and prostate cancer, while ovarian cancer and renal cancer cell lines are the least sensitive. In this analysis (Hu & Yao, 2001), MCF7 cell line is described as moderately sensitive in respect to tests with steroidalSaponins. Here we found that IC_{50} leaf Saponin is 6.25 µg/ml and IC_{50} of Seed Saponin extract is 12.5 µg/ml. Considering the data of Hu *et al.* for a moderate sensitivity of MCF7 and our MTT assay results, it can be assumed that *Tribulus* of Indian origin could have even stronger antitumor effect on cancer cell lines with higher sensitivity to steroid Saponins. Similar to our results,Angelova*et al.*,had got the IC_{50} of 15 µg/ml.Comparable to the MTT assay results, the SRB assay results showed that seed methanolic extract was far more effective than leaf methanolic extract and similarly seed Saponins were more effective than leaf Saponins.4 different concentrations of Methanolic extracts showed no activity in LDH assay which were previously showing activity in above assays. While rest all extracts showed profound cell cytotoxicity comparable with the MTT and SRB assay results. MTT assay is based on activity of NAD(P)H-dependent oxido-reductase enzyme which convert MTT to formazan in viable cells while LDH measures the released LDH enzyme from damaged plasmamembrane of the cells. Similarly, SRB assay measures the amount of protein present in viable cells. Thus, MTT and SRB assay may shows cytotoxicity in the cells having low metabolic rates and not necessarily the cells undergoing apoptosis while LDH assay accurately points towards cell death by measuring the amount of LDH released from apoptotic cells and not the viable cells. Our results indicate that LDH is much more sensitive than MTT and SRB assay.

V. Conclusion

In Summary, from the present study it can be concluded that methanolic and Saponin extract of *Tribulus terrestris* possess cytotoxicity against Human breast cancer cells MCF-7 in all the cytotoxic assays studied. In all assay, the Saponin fractions have shown higher activity as compared to crude methanolic extracts. Thus, this finding directs further investigation of molecular mechanism of action and evaluating chemical structure of Saponins.

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