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Research Article

ZIZYPHUS MAURITIANA (INDIAN JUJUBE/BERI SPIKES) OF BIKANER, RAJASTHAN OF INDIA - AN ANTI-CANCEROUS DRUG FORMULATION FOR BLOOD CANCER WITH VALIDATED IC50-CALIBRATION VIA FTIR, FESEM EDAX

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ABSTRACT

In order to analyse the bioactive constituents of spikes of *Zizyphus Mauritiana*/ Beri spikes of Bikaner, Rajasthan, Fourier Transform Infrared Spectroscopy (FTIR) and added Field Emission Scanning Electron Microscope with Energy Dispersive X-ray analysis (FESEM-EDAX) were carried out initially. The authors too demonstrated Cell line studies via MTT Assay for MOLT4 (Blood cancer) in different concentrations and found Beri spikes as toxic with Validated IC50 via Graph Prism Software. Concluded by the result, phytochemicals present in the spikes reflects it as bioactive compound which may act as antimicrobial, anti-cancerous, antiviral, and anti-inflammatory agents. On an all, Indian Jujube is all set to form a traditional medicine because of the effects it got via cell death when experimented for in-vitro trials on Blood Cancer cells.

INTRODUCTION

It is one of the important plant species of Rajasthan which is having enormous functionalities of traditional Indian medicinal weightage, yet unexplored. Its ethanolic/methanolic extracts covering root, spikes, bark, and seeds have shown anticancer activity in cell culture studies. They act against multiplication of cancerous cell division and cause them death. Apart from anticancer activity, it may possess antioxidant action which might help in managing cancer. It is too useful for people having insomnia as the sedatives from *Zizyphus Mauritiana* results in drowsiness. The spikes are a good source of compounds such as polysaccharides, flavonoids and triterpenic acids that are having antioxidant properties. Antioxidants are substances that may delay or prevent different types of cell damage including damage caused by free radicals. Beri contains minerals [3,4] such as copper, manganese, magnesium, and potassium that improves osteoporosis and overall bone health.

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Unsaturated fatty acids and Vitamin C present in it have an anti-cancer and added anti-aging effects for homosapiens. It antagonizes endothelin-1 receptors and decreases blood pressure. Its fruits and seeds are used for medicinal purposes in traditional Chinese medicine. It possesses anxiety-reducing and other sedative properties. It too regenerates mental health and having good effects on constipation. It is already proved that in ibuprofen induced nephrotoxicity rats, its intake extract from fruit, around 500mg/kg improved kidney function by declining the levels of urea and creatinine. Because of the association of flavonoids and phenolic acids, jujube fruits exhibit therapeutic treatment in liver failure. Because of the part of high- dietary fiber content, it is very useful for diabetics too having been associated with blood thinning effects. On an all, jujube is an antioxidant rich superfood.

Ayurvedic Uses of its Spikes: Here, in this study, we focus on the spikes of Indian jujube with anticancer testing on MOLT4- Blood cancer via MTT Assay Cell line in-vitro studies via Biotechnology. As according to the holistic approach of Ayurveda, its spikes has been extensively used as a potential herbal medicine from decades. According to the literature, it has been observed that its seeds and leaves are valued for its incredible healing properties for treating bleeding disorders, burning sensations and excessive thirst. The

antipyretic activity[5,6,7] of BERI leaves helps in mitigating fever. On the other hand, its fruit powder combined with honey is used as a protection mask to heal skin infections due to having wound healing characteristics. Dried ber balances the *Kapha* and *Vata* *dosha*, while sour ber pacifies the same. It comprises 18 of the 24 essential amino acids that promote development and growth. It helps against chronic health conditions like cancer, diabetics, and heart diseases.

The nutritional values of a Raw Jujube contain

Vitamins	
Vitamin A	11.12 IU
Vitamin C	19.3 mg
Thiamine	0.0 mg
Riboflavin	0.0 mg
Niacin	0.3 mg
Vitamin B6	0.0 mg
Vitamin B12	0.0 mcg
Minerals	
Calcium	6 mg
Iron	0.13 mg
Magnesium	3 mg
Phosphorus	6 mg
Potassium	70 mg
Sodium	1 mg
Zinc	0.01 mg
Energy	22 Kcal
Carbohydrates	5.66 g
Protein	034 g
Total Fat	0.06 g
Cholesterol	0 mg

As according to the U.S.D.A- United States Department of Agriculture

Study Area Topological Map- Bikaner, Rajasthan of India- Where sample have been collected.

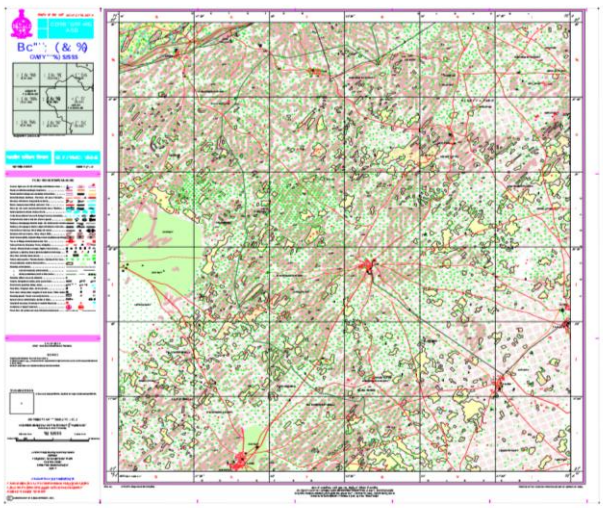


Figure 1: Elucidates the geographical toposheet of Bikaner, Rajasthan, India from samples have been collected in the month of October, 2022. Topological map assigned from Survey of India

MATERIALS AND METHODS

Plant sample spikes were obtained from Bikaner, Rajasthan, India in the month of October 2022 and deposited to Lab No. 14 of Dept of Earth Sciences, Annamalai University. Binocular microscopic pictures were taken. In the process of plant extraction, material (BERI spikes) was dried and powdered to 100gm, preheated sample was placed in Soxhlet apparatus for 2 to 3 days, the samples were extracted in distilled cow urine, DMSO, methanol, ethanol, chloroform, and ethyl acetate for different tests. Remains collected via extraction were sterilized and put into vials of different types for different analysis.

Fourier Transform Infrared Spectroscopy (FTIR)- These spectrometers are just like NDIR analyzers, i.e., the fact that many gases absorb IR radiation at species- specific frequencies. It is a disperse method that gestures the measurements are performed over a broad spectrum instead of a narrow band of frequencies. This spectroscopy takes advantage of how IR light changes the dipole moments in molecules that corresponds to a specific vibration energy. Fast Fourier Transform Method on which the modern FTIR Spectroscopy is based was introduced by Turskey and Cooley in 1965. First FTIR spectrum was recorded by Peter Fellgett in 1949. This spectrometer uses infrared light to scan test samples with an observation on chemical properties. It is a quick analysis to identify compounds with functional groups and classes. FTIR provides higher wavelength accuracy with widest possible wavelength range.

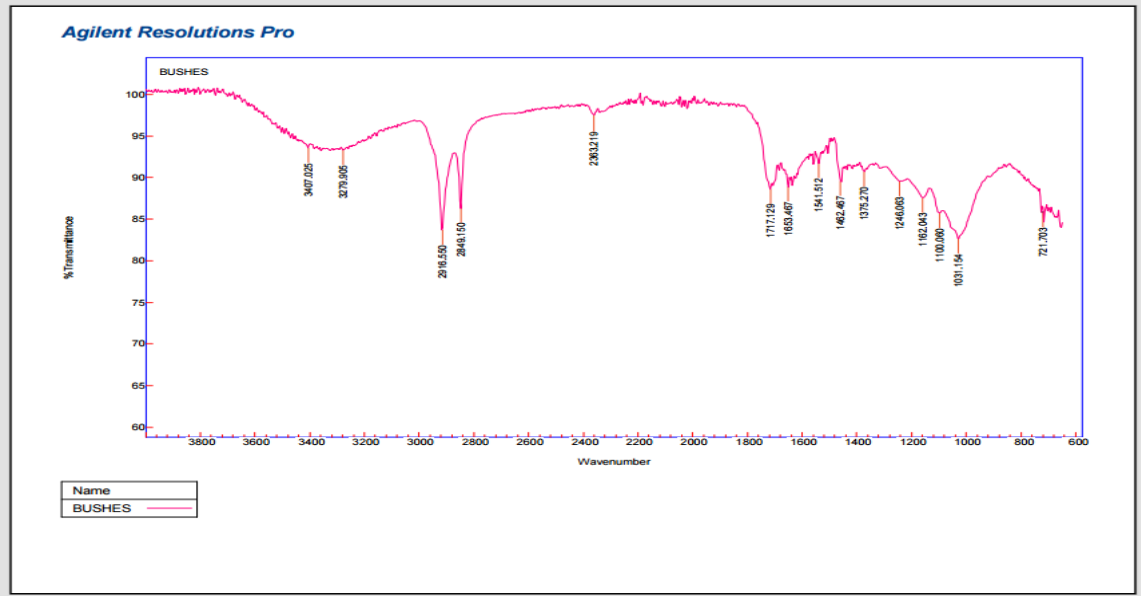


Figure 2: IR representation of Beri Spikes with different peak values and transmittance

IR Spectrum Table

Table 1: Conclusion table from Infrared Spectrum

Wave number (Highest Peaks)	Transmittance %	Functional Class	Stretching Vibration
3407.025	94	Alcohols / Phenols	O-H (free), usually sharp
3270.905	94	Alcohols/Phenols	O-H (H-bonded), usually broad
2916.550	84	Alkanes	C-H stretch

Field Emission Scanning Electron Microscope Energy Dispersive X-ray Spectroscopy (FESEM EDAX)

The primary principle of EDAX is a generation of X-rays from a specimen through the electron beam. This technique can also be used measure the energy of emitted rays. FESEM provides probing beams at high as well as low electron energy. FESEM EDX investigations enable us to reveal the correlation between morphological changes and elemental composition. Aging processes were clarified by learning changes in the elemental composition to the fiber core in cross-sections.

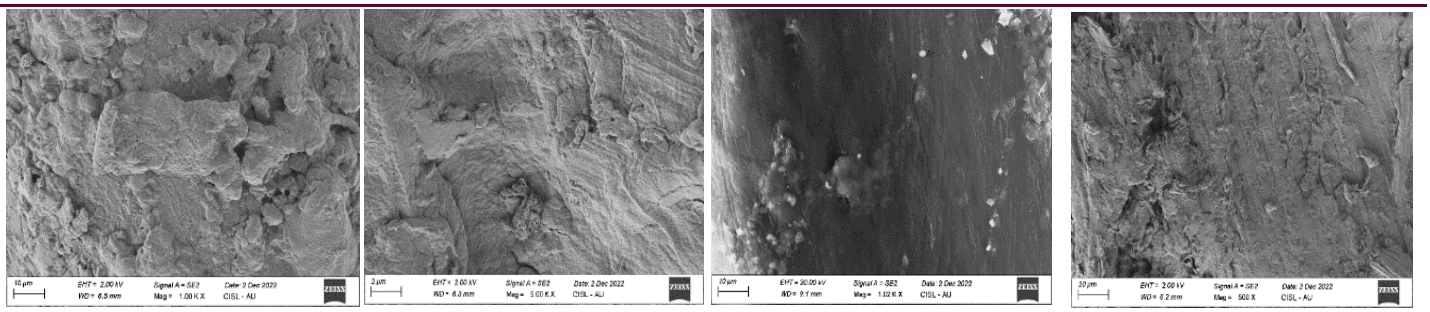


Figure 3: FESEM images with different magnifications

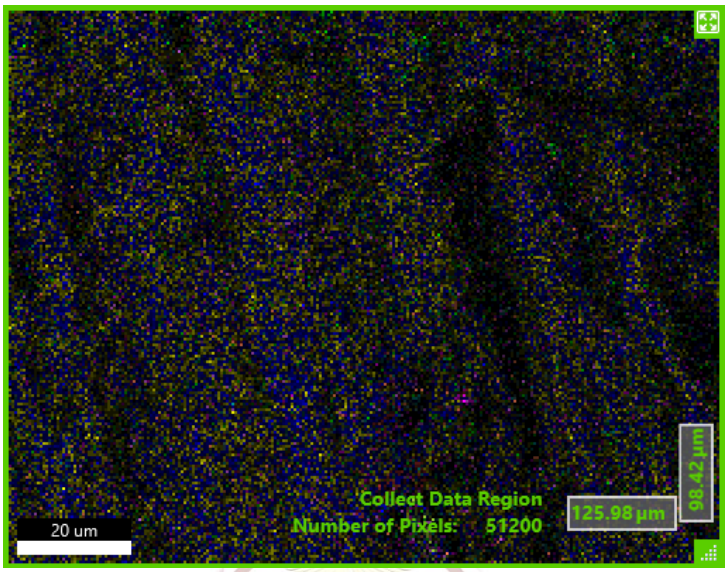


Figure 4: Amalgamation of different elements via FESEM Mapping altogether

Element	Weight %	MDL	Atomic %	Error %	Net Int.	R	A	F
C K	51.86	0.1	59.40	9.77	1170.26	0.9341	0.1472	1.0000
O K	46.61	0.06	40.07	10.30	1650.60	0.9422	0.0905	1.0000
Mg K	0.30	0.02	0.17	9.73	58.60	0.9540	0.3979	1.0021
P K	0.23	0.01	0.10	5.96	59.10	0.9612	0.7670	1.0076
K K	0.28	0.02	0.10	5.98	53.10	0.9692	0.9398	1.0260
Ca K	0.20	0.02	0.07	7.52	32.65	0.9710	0.9555	1.0315
Mn K	0.09	0.03	0.02	16.92	8.85	0.9792	0.9899	1.1171
Fe K	0.01	0.03	0.00	62.91	1.06	0.9808	0.9923	1.1420
Cu K	0.04	0.04	0.01	60.00	2.48	0.9852	0.9954	1.2568
Zn K	0.03	0.05	0.01	60.59	1.46	0.9867	0.9971	1.3049
Mo L	0.34	0.03	0.05	7.89	35.68	0.9640	0.8364	1.0029

Figure 5: EDAX results of elemental composition with Carbon and oxygen at maximum

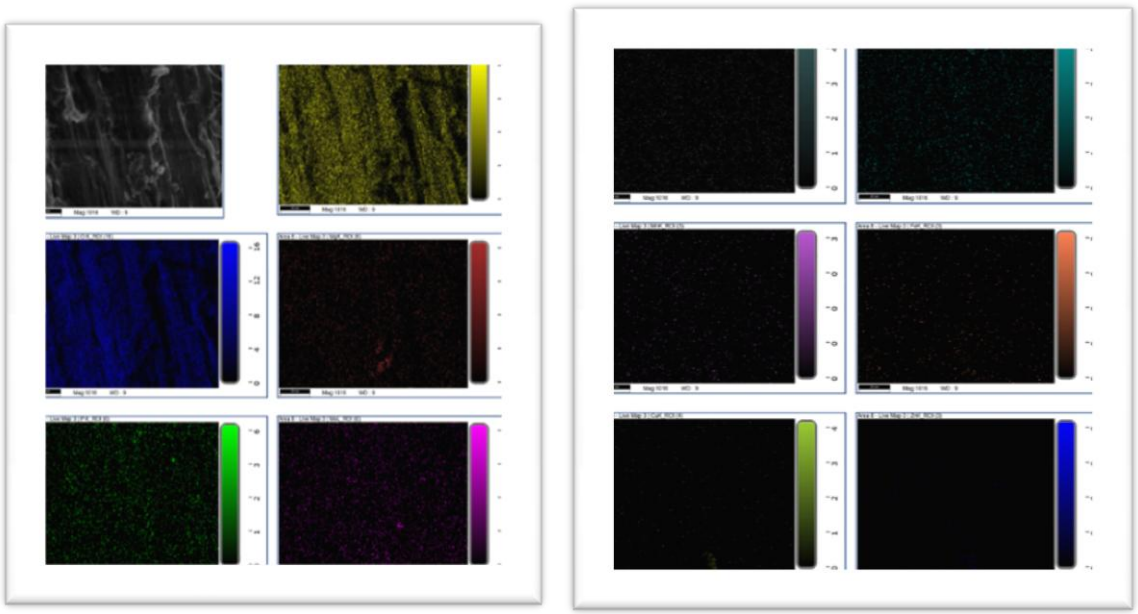


Figure 6- EDAX mapping of different elements individually

Binocular Images

Figure 7: Dried spikes captured under microscope

Video URL link from where the samples have been collected-
https://drive.google.com/file/d/1vNzBTYzSHbSUTOY4VnybyLHtCjxYmeyI/view?usp=share_link

Figure 8: Indian Jujube spikes - raw leaves captured via camera, immediately after collection from Khejri tree there from Bikaner, Rajasthan, India

Biotechnology

MTT Assay for Cell Cytotoxicity

Principle- (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) MTT assay id the capability of mitochondrial dehydrogenase enzyme from viable cells to cleave the rings from tetrazolium pale yellow MTT and hence to form blue colored formazan crystals that are largely impermeable to cell membranes, thus outputting in its accumulation within healthy cells. By the addition of detergents like DMSO, solubilization of cells results in the liberation of crystals that are soluble. Number of surviving cells is directly proportional to the level of formazan so created. We can quantify the color using a multi-well plate reader.

Materials Required – Dulbecco's modified eagle medium (DMEM) [1,2], antibiotic solutions and Fetal Bovine serum (FBS) were brought from Gibco, USA. Dimethyl sulfoxide (DMSO) and 3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide (MTT)(5mg/ml) were landed from Sigma, USA. 1X Phosphate Buffer Saline (PBS) was from Himedia, India. Wash beakers and Tissue culture plates with 96 wells were from Tarson, India.

PROCEDURE

Cell Culture- MOLT 4 cells (Blood cancer cells) were purchased from NCCS, Pune, and were cultured in liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100ug/ml of penicillin and 100 µg/ml of streptomycin and are maintained within an atmosphere of 37 degrees Celsius along with 5% of CO_2.

MTT Assay- Beri spikes- Targeted sample was tested for *in vitro* cytotoxicity, using MOLT 4 cells by MTT assay. Briefly, the cultured MOLT 4 cells were harvested by trypsinization, pooled in a 15ml tube. At a density of $1*10^5$ cells/ml cells, cells were plated with cells/well (200µL) into 96- well tissue cultured plate in DMEM containing 10% of FBS and 1% of antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the 5 sample in a serum free DMEM medium. Each sample was in replication for three times and at 37°C, cells were incubated in a humidified 5% CO_2 for 24 hours. After incubation period, MTT (20µL of 5mg/ml) was inserted into each well and cells were incubated for another 2 to 4 hours until purple precipitates were clearly seen under inverted microscope. Finally, the medium along with MTT (220µL) were aspirated off the wells and washed after that with 1X PBS (200µl). Further-after, DMSO (100 µL) was added to dissolve formazan crystals, and the plate was shaken for 4 to 5 minutes. Absorbance for each well was measured at 570 nm using micro plate reader (THERMO FISHER SCIENTIFIC, USA) and IC50 and Percentage cell viability was calculated using Graph Pad Prism 6.0 Software, USA [10,11].

Formula Cell viability % = Test OD/Control OD X 100

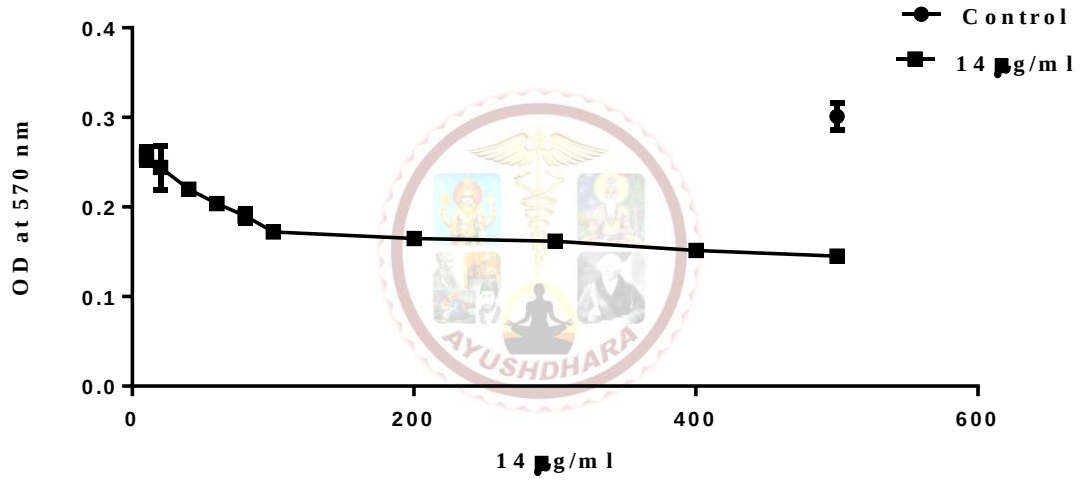
RESULT

Table 2: Elucidating different concentrations with OD values at 570nm

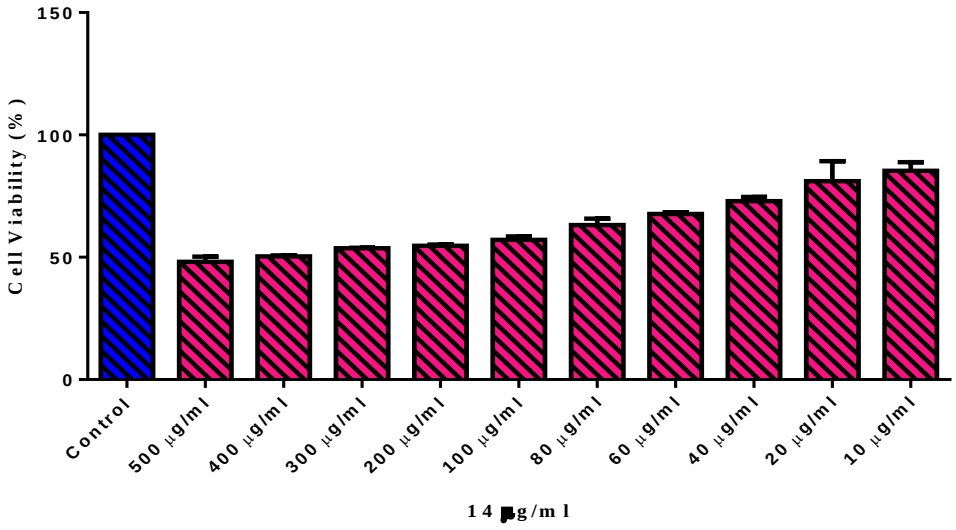
S. No	Tested-sample, concentration (µg/ml)	OD Value at 570 nm (In triplicates)		
1.	Control	0.293	0.318	0.292
2.	500 µg/ml	0.138	0.147	0.15
3.	400 µg/ml	0.151	0.151	0.153
4.	300 µg/ml	0.162	0.162	0.161
5.	200 µg/ml	0.163	0.166	0.165
6.	100 µg/ml	0.168	0.172	0.176
7.	80 µg/ml	0.198	0.19	0.182
8.	60 µg/ml	0.203	0.206	0.202
9.	40 µg/ml	0.215	0.219	0.225
10.	20 µg/ml	0.217	0.25	0.265
11.	10 µg/ml	0.261	0.245	0.265

Table 3: Different concentrations with cell viability and mean

S. No	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	45.84718	48.83721	49.83389	48.17276
3.	400 µg/ml	50.16611	50.16611	50.83056	50.3876
4.	300 µg/ml	53.8206	53.8206	53.48837	53.70986
5.	200 µg/ml	54.15282	55.1495	54.81728	54.70653
6.	100 µg/ml	55.81395	57.14286	58.47176	57.14286
7.	80 µg/ml	65.78073	63.12292	60.46512	63.12292
8.	60 µg/ml	67.44186	68.43854	67.10963	67.66334
9.	40 µg/ml	71.42857	72.75748	74.75083	72.97896
10.	20 µg/ml	72.09302	83.05648	88.03987	81.06312
11.	10 µg/ml	86.71096	81.39535	88.03987	85.38206



Plot 1: OD at 570nm with 5µg/ml concentrations



Plot 2: Cell viability at different concentrations

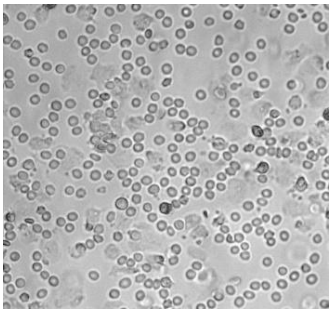
IC50 Value of tested sample: 62.22µg/ml

log(inhibitor) vs. normalized response -- Variable slope		
Best-fit values		
LogIC50		1.794
HillSlope		-1.637
IC50		62.22
Std. Error		
LogIC50		0.02558
HillSlope		0.1639
95% Confidence Intervals		
LogIC50		1.742 to 1.846
HillSlope		-1.973 to -1.301
IC50		55.15 to 70.19
Goodness of Fit		
Degrees of Freedom		28
R square		0.9440
Absolute Sum of Squares		1916
Sy.x		8.271
Number of points		
Analyzed	3	30

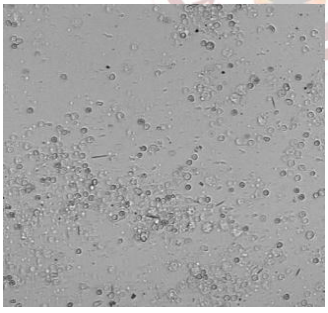
Table 3: Statistical outputs via Elisa, Graph Prism with IC50 as 62.22

Morpholgy

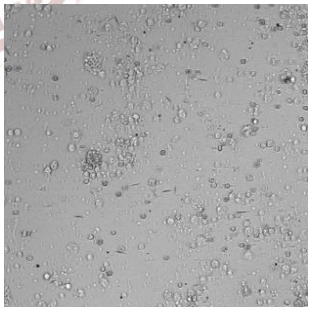
Images of control cells and treated cells at different concentrations



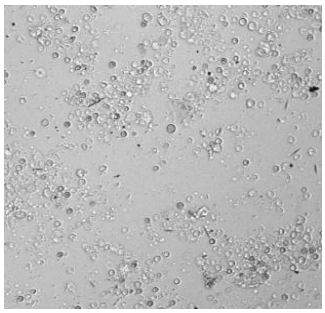
Control cells



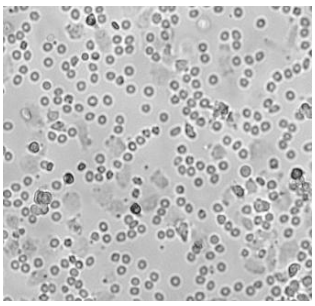
500µg/ml



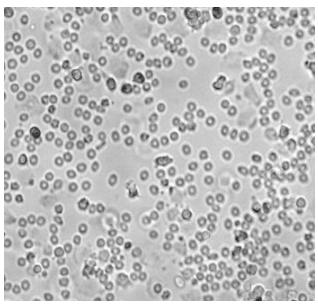
300µg/ml



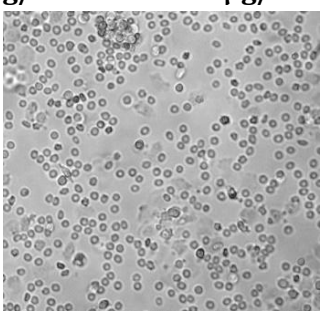
100µg/ml



60µg/ml



20µg/ml



10µg/ml

DISCUSSION

The authors demonstrated and designed the entire experiment in order to find the anti-cancerous properties of phytochemicals present in the sample- BERI SPIKES. To begin with, the authors downloaded the topographical map of Bikaner, Rajasthan, India from Survey of India and studied the seasonal variations of different Land Use Land Pattern via satellite data so provided by SOI. Samples were arrived easily and stored in laboratory via sampling from location at particular time of particular season.

The authors performed FTIR- Fourier Transfer Infrared Spectroscopy to find the functional group/s where the different peak falls.

Samples were isolated, soxhelated and extracted using Distilled cow urine at first when was going through cold isolation for 4 to 6 days. MTT Cell line studies for blood cancer via biotechnological approach were performed and it is observed that toxicity was present in the sample and was a look alike of anti-cancer medication. May be the compounds present in the vials, in sample are having different importance that are not only confined to cancer but too to other disabilities/diseases. Furthermore, the phytochemicals present in them that are bioactive and have toxicity reflects the medicinal importance of *Zizyphus Mauritiana* spikes.

Proceeded with Blood cancer for *in-vitro* experimentation, cell lines of MOLT4 cancer cells was given treatment with our drug in different concentrations and we found that *Beri spikes* is one of the best fit to be an anti-cancer agent which simply means that we can use it in the treatment for human blood cancer as nano medicine with different solvents, let it be distilled cow urine, DMSO D6 etc which proves that yes, Indian Jujube roots are having anti-cancerous properties which can be used as in a form of pellet or vaccine or drug etc whether Ayurvedic, homoeopathy, allopathy, or Unani or may use as a Nano powdered in Radiotherapy for blood cancers.

CONCLUSION

The authors concluded that Jujube spikes when interfered with distilled cow urine and DMSO D6 as solvents, results as a suitable agent being capable of eradicating blood cancer. The authors too demonstrated the anti-cancer toxicity of ZIZYPHUS MAURITIANA SPIKES via MTT Assays for in-vitro cell lines of human blood cancer and found amazing results which itself is a proof that it can be used as a drug with few solvents to treat cancer patients. In order to re-examine, the authors performed anti-oxidation, MTT Assays thrice, in triplicates as part of analysis and found drastic cell death and concluded it as an anti-cancerous agent.

Data Availability- The authors confirm that the data supporting the findings of this study are available within this article.

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Abbreviations

MTT- 3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide

FTIR- Fourier transform infrared spectroscopy

IC50- Inhibitory Concentration at 50%

FESEM- Field Emission Scanning Electron Microscope

EDAX- Energy Dispersive X-ray Spectroscopy

DMSO- Dimethyl Sulfoxide

FBS- Fetal Bovine Serum

CO_2- Carbon Dioxide

DMEM- Dulbecco's modified Eagle medium

PBS- Phosphate Buffer Saline

MOLT 4- Human T lymphoblast, acute lymphoblastic leukaemia (cell line slides)

OD- Optical density

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