WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 7, Issue 7, 781-795 Research Article ISSN 2278 – 4357

DEVELOPMENT OF HERBAL FORMULATION WITH *AJWA* SEED (*PHOENIX DACTYLIFERA* L.) EXTRACT AND ZAM-ZAM WATER FOR *INVITRO*-AND *INVIVO* ANTICANCER ACTIVITY

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Article Received on
30 April 2018,

Revised on 20 May 2018,
Accepted on 10 June 2018

DOI: 10.20959/wjpps20187-11894

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ABSTRACT

The present research work of Herbal Formulation With Ajwa Seed (Phoenix Dactylifera L.) Extract And Zam-Zam Water For Anticancer activity is an effective formulation from the pioneer research and discussion. As well as literature survey, the possibility of the study is fine. The parameters and methodology for the research work is made initially by plant authentifications. Phoenix Dactylifera was authentified by respective authority. The seeds of riped fruit was crushed, powdered and examined for number of evaluation studies, which includes physical characteristic studies like smell, taste, colour, solubility studies and loss on drying. The dried seeds of Phoenix Dactylifera ethanolic extract was prepared by soxhlet apparatus by using ethanol (95%v/v)as a solvent. Phyto chemical screening also performed to determine presence of active constituents. The advanced studies also performed with the help of HPTLC finger printing method for qualitative evaluation of seeds compared sample with standard, and result proved that active constituent present in the sample Phoenix Dactylifera is similar as standard. and the acute toxicity study was performed by OECD Guidelines 423 and therapeutic index was determined, which were correlated standard parameters. The cell line studies was performed

by selected cell Seven cell lines HT-29 (Human colon cancer cell lines), A 431(Human skin cancer cell lines), HL-60 (Human leukemia cell lines), MCF-7 (Human breast cancer cell lines), and Human Epidermoid Larynx Carcinoma cell line (Hep 2). A 549 (Human lung cancer cell lines), HeLa (human cervical cancer) Dalton's lymphoma cells (mouse ascites tumor) which includes MTT assay, SRB assay, LDH assay etc. The in vivo cancer studies performed in rats. the result given by all evaluation parameters are satisfactory by achieving all the experimental criteria. The optimized seed powder formulated pharmaceutically for formulations were confirmed by six trials. The invtro studies were performed with the help of analytical studies. The stability studies also performed for six month. For the formulation. zam zam water was selected as vehicle because of abundant source from natural and certain anti oxidant effect. In the light of the above points and considering the importance of natural drugs, the present work on the investigation of the plant namely *Phoenix dactylifera* L.Family *Arecaceae* reveals The generation of new therapeutic agents for the Anti-Cancer.

KEY WORDS: *Phoenix dactylifera* L, HPTLC finger printing method, Zam-Zam Water, MTT assay.

INTRODUCTION

Cancer is a disease of multicellular organisms[1] characterized by uncontrolled multiplication of subtly modified normal human cells.[2] Cancer is a leading cause of death all over the world and represents a major public health burden.[3] They constitute the second cause of mortality behind cardiovascular diseases in developed countries and the third after infectious and cardiovascular diseases in developing countries.[4] Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered.[5] All of the drawbacks presently associated with available chemotherapeutic agents are impetus for the search for newer, more efficacious, and better tolerated drugs. Natural products, especially the plant kingdom, offer an inexhaustible reservoir for investigation. Plants have a long history of use in the treatment of cancer[6,7] and the interest in nature as a source of potential chemotherapeutic agents continues.[8] Fruits of the date palm (Phoenix dactylifera L. Arecaceae) are very commonly consumed in many parts of the world and are a vital component of the diet in most of the Arabian countries. Date is one of the oldest known fruit crops and has been cultivated in North Africa and the Middle East for at least 5000 years.[9] The earliest record from Iraq (Mesopotamia) shows that date culture was probably

established as early as 3000 BCE. Because of the long history of date culture and the wide distribution and exchange of date cultivars, the exact origin of the date is unknown, but it most likely originated from the ancient Mesopotamia area (southern Iraq) or western India.[10] Seeds of *Phoenix dactylifera* L against various Human cancer cell lines. One of the miracles of Zamzam water is its ability to satisfy both thirst and hunger. More recently, in the last few decades, samples of Zamzam water have been collected by scientists and they have found certain peculiarities that make the water healthier, like a higher level of calcium.[11]

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, reagents and solvents used in the study were of analytical grade.

Solvents

95% Ethanol, Methanol, Diethyl ether, Chloroform, DMSO (Dimethyl sulphoxide).

Reagents

Trypan blue (Hyclone, Lot no: JRH27098), EDTA (MP Biomedicals, Lot No: 6941H), Trypsin (Invitrogen, Lot No: 1376596), MTT (Roche applied sciences, Cat. No: 11465 007 001).

Media

RPMI-1640 (Sigma Aldrich Ltd. Mumbai), FBS (Fetal Bovine Serum) (Bioclot, Lot No: 07310).

Equipments

Fluorescence inverted microscope (Leica DM IL), CO2 incubator (RS Biotech, mini galaxy A), ELISA plate reader (Lab system Multiscan), Melting point apparatus (Veego Melting Point apparatus-VMP-PM), IR (Shimadzu-8400S FTIR spectrophotometer), APCI-MS (Atomic Pressurized Chemical Ionization-Mass Spectroscopy) (Varian Inc, USA-410 Prostar Binary LC with 500 MS IT PDA Detectors spectrophotometer), ^{1}H NMR (Varian Mercury YH-300 MHz ^{1} H NMR spectrophotometer).

Cancer cells: Five cell lines, HL-60 (Human leukemia cell lines), HT-29 (Huma colon cancer cell lines), MCF-7 (Human breast cancer cell lines), A 431(Human skin cancer cell lines) and A 549 (Human lung cancer cell lines), Human Epidermoid Larynx Carcinoma cell line (Hep 2). were procured from the Amala Cancer Research Institute, Kerala, India.

Preparation of the extract/ drug: The granulated dried seeds of *Phoenix dactylifera* L (500gm)was packed in a Soxhlet apparatus and subjected to continuous hot percolation for using 450 ml of ethanol (95% v/v) as solvent. The extract was concentrated to dryness under reduced pressure and controlled temperature and dried in a desiccator (yield 75 g, 15% w/w). The extract was suspended in Zamzam water and used for further experiments.

Preliminary phytochemical screening

The extract was screened qualitatively for the presence of various groups of phytoconstituents using different chemical tests.[5, 6]

Instrumentation

In the present work Camag HPTLC system equipped with Linomat 5 applicator, twin trough chamber (20x10cm, 0.2 mm thick) size, TLC scanner 3, Reprostar 3 with 12bit CCD camera for photo documentation, controlled by WinCATS- 4 software were used. All the solvents used were of high grade obtained from MERCK. All weighing was done on Precisa XB 12A digital balance.

Preparation of standards and sample solution:

2mg in 10 ml → 0.2 mg /ml → 200 µg/1000µl →0.2µg/µl

Mobile phase

The organic solvents such as toluene**:** ethyl acetate**:** formic acid**:** methanol (3:6:1.6:0.4) was used as a mobile phase.

Chamber used for mobile phase

Camag twin trough chamber (20 x 10 cm).

Chamber saturation

Chamber saturation was done for 5 minutes.

Stationary phase

TLC aluminum sheet precoated with silica gel 60 F254, (20x10cm) was used as stationary phase, obtained from MERCK.

Procedure

The Ethanolic seeds extract solutions were prepared. The TLC plate was activated by heating at 1200C for about 30 min prior to use. Ethanolic extract solution (2 µl), standard solution (0.2µg/µl) were applied in duplicate, as tracks 8, with a band length of 6.0 mm each on a precoated silica gel 60 F254 TLC plate, with Linomat V applicator using a Hamilton syringe (100µl).Mobile phase used toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4). No prewashing of the plate was done. Chamber saturation time was 5 minutes. The TLC plate was kept for development to a migration distance of 77 mm. Post derivatization had been done with vanillin-phosphoric acid. The derivatized plate was dried in hot air oven at 60 ^{0}c for 5 minutes and scanned at 254 nm, band length 6.0 mm, slit dimension 6.00x0.45mm, micro, scanning speed (20mm/s) and source of radiation was Deuterium and Tungsten lamps respectively. The Rf and peak area of the spots were interpreted by using the software. The derivatized plate was photo documented under 254 nm light using Camag Reprostar 3, equipped with 12 bit CCD camera.[7,8]

EVALUATION OF *INVITRO* ANTI-CANCER ACTIVITY[12-14] Screening methods of anticancer activity Cell lines and culture conditions

Five cell lines, HL-60 (Human leukemia cell lines), HT-29 (Huma colon cancer cell lines), MCF-7 (Human breast cancer cell lines), A 431(Human skin cancer cell lines) and A 549(Human lung cancer cell lines), Human Epidermoid Larynx Carcinoma cell line (Hep 2). were procured from the Amala Cancer Research Institute, Kerala, India. These cell lines were cultured in RPMI-1640 medium. The media were supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100units/ml) and streptomycin (100g/ml). The cultures were maintained in a humidified 5% CO2 incubator at 370C and the cells were sub cultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h before use.

Subculture of adherent cell lines (HT-29, MCF-7, A-549 and A-431)

Cultures were viewed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with FBS without Ca2+/Mg2+ using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1ml per 25 cm2 of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the CO2 incubator and left for 2- 10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small

volume of fresh serum containing HT-29, MCF-7, A 549 and A 431 medium respectively. 100-200ìl was removed to perform a cell count. The required numbers of cells were transferred to a new labeled flask containing pre-warmed HT-29, MCF-7, A 549 and A 431 medium and incubated as appropriate for the cell line.

Subculture of adherent cell lines (Hep 2)

Cultures were viewed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without Ca 2+/Mg2+ using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1ml per 25 cm2 of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the CO2 incubator and left for 2- 10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing Hep-2 medium. 100-200ìl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed Hep-2 medium and incubated as appropriate for the cell line.

MTT assay

MTT Colorimetric assay is based on the capacity of Mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. MTT assay was employed to assess cell proliferation. Viable cells were seeded into 96- well microtitre plates at 2× 104 cells/well in RPMI-1640 medium supplemented with FBS (fetal bovine serum), 100units/ml penicillin, 100ìg/ml streptomycin, and were cultured in a humidified atmosphere of 5% CO2 and 95% air at 370C. 150ìl of cell suspension was cultured with 10ìl of various concentrations i.e. 10, 20, 40 and 80ìg/ml18 of the ethanolic seeds extract *Phoenix dactylifera* L respectively dissolved in DMSO (dimethyl sulphoxide) as solvent and incubated for 48h. Similar solutions containing the same concentrations of cyclophopamide were also be prepared and served as standard solutions. Control cells were incubated in RPMI-1640 medium only. Wells containing only media were considered as a blank. All cyclophosphamide, ethanolic extract and dilution doses were tested in triplicates. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium

reductase system to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. 10 ìl of MTT labeling mixture was added and incubated for 4 h at 37°C and 6.5% CO2. After 4h, 100 ìl of solubilization solution was added in each well. After 48h incubation at 370C temperature and 5% CO2, the absorbance of soluble formazan product produced by viable cells was measured at 450nm using ELISA plate reader. Reference wavelength used was 630 nm. Percentage inhibition of the cell proliferation by cyclophosphamide, ethanolic seeds extract *Phoenix dactylifera* L against all cell lines was calculated using the following formula,

$$\% \quad \textbf{Cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where**,** At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells).

Sulphorodamine B assay[15]

Sulphorodamine B (SRB) is a bright pink Aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude.[15-16] The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 ìl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37oC for 72 hours in 5% CO2 incubator and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, 25 ìl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form a overall concentrations 10%. The plates were incubated at 4oC for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100ìl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100 ìl of 10mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a

wavelength of 540nm.

Statistical analysis

The results of various studies were expressed as mean ± SEM and analyzed statistically using one way ANOVA followed by Dunnets Test to find out the level of significance. Data were considered statistically significant at minimum level of $p < 0.05$.

RESULTS AND DISCUSSION

The preliminary phytochemical analysis of fractions of *Phoenix dactylifera L.* shows the presence of steroids, alkaloids, flavonoids, glycosides, saponins, tannin and carbohydrate.

Table No 1: Preliminary phytochemical screening.

Constituents	**Test**	***Phoenix dactylifera* L.,**
Carbohydrates	Molisch Test Fehling's Test Benedict's test: Barfoed's test:	+ + + +
Alkaloids	Dragendroff's Test Wagner's test Mayer's Test Hager's Test	+ + + +
Steroids and Sterols	Liebermann Burchard test Salkowski test	- +
Glycosides	Legal's test Baljet test Borntrager test Killer Killani test	+ + + +
Saponins	Foam test	+
Flavonoids	Shinoda test	+
Tri-terpenoids	In the test tube, 2 or 3 granules of tin+2ml of thionyl chloride solution and test solutionis added.➔Pink color	+

HPTLC fingerprinting of *Phoenix dactylifera L.* seeds extract

The ethanolic seeds extract of *Phoenix dactylifera L.* were subjected to generate HPTLC fingerprinting profile represented as the chromatogram. The solvent system used in the investigation was found to give compact spots for extracts at different Rf values table 9-12

Table No 2: Observation of Rf values and % area of the chromatogram of rutin gallic acid at 254nm (std).

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	22.8	0.02	89.9	8.13	0.07	1.4	4.57	4.57	Unknown*
2	0.08	0.3	0.13	147.1	13.30	0.21	14.2	1.26	14.26	**Rutin**
3	0.21	14.4	0.24	45.0	4.07	0.25	43.0	1.84	1.84	Unknown*
4	0.25	43.5	0.26	54.1	4.89	0.32	0.0	1.82	1.82	Unknown*
5	0.37	9.1	0.38	10.5	0.95	0.41	0.1	0.30	0.30	Unknown*
6	0.45	3.2	0.46	12.3	1.11	0.49	1.6	0.33	0.33	Unknown*
7	0.55	1.6	0.59	19.7	1.79	0.60	19.2	0.66	0.66	Unknown*
8	0.64	14.3	0.70	320.3	28.96	0.74	123.4	22.06	22.06	**Gallic acid**
9	0.74	124.4	0.84	391.1	35.36	0.96	0.1	53.99	53.99	**Quercetin**
10	0.97	0.8	0.98	15.9	1.43	0.98	3.7	0.18	0.18	Unknown*

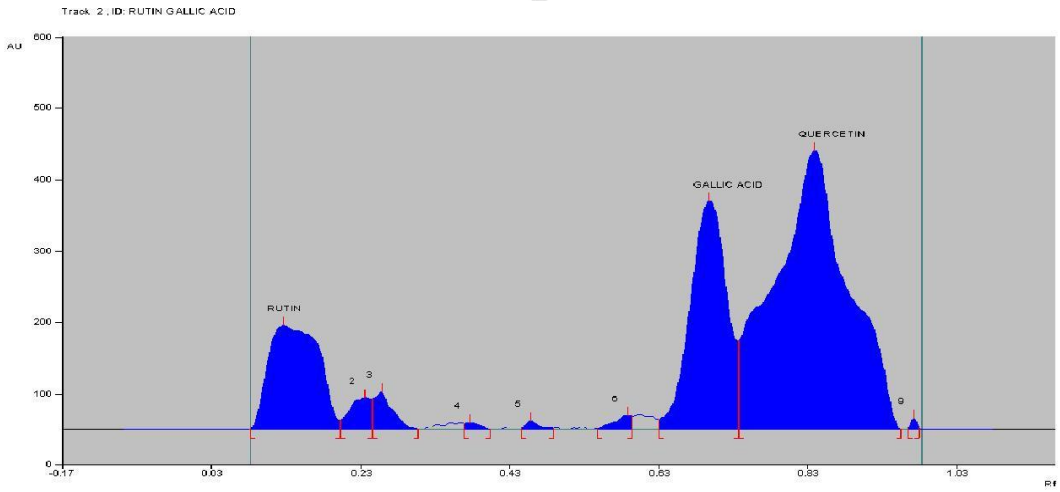

Fig. 1: Observation of Rf values and %area of the chromatogram of rutin gallicacid at 254nm (std).

Table No. 3: Observation of Rf values and %area of the chromatogram of Quercetin at 254nm (std).

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	4.5	0.02	117.0	13.98	0.07	40.7	3590.9	13.87	Unknown*
2	0.18	17.3	0.19	19.4	2.32	0.24	0.0	417.1	1.61	Unknown*
3	0.28	3.2	0.30	16.1	1.93	0.32	0.9	171.7	0.66	Unknown*
4	0.32	2.2	0.33	18.6	2.22	0.34	0.0	90.3	0.35	Unknown*
5	0.38	0.0	0.40	12.1	1.44	0.41	2.9	128.5	0.50	Unknown*
6	0.41	3.1	0.44	20.3	2.43	0.47	12.5	396.1	1.53	Unknown*
7	0.47	13.6	0.47	23.1	2.76	0.49	2.5	161.9	0.63	Unknown*
8	0.50	0.6	0.51	19.0	2.27	0.54	0.0	199.5	0.77	Unknown*
9	0.55	0.7	0.56	22.0	2.63	0.58	2.6	236.9	0.91	Unknown*

10	0.70	16.3	0.74	37.2	4.44	0.75	26.7	656.5	2.54	Unknown*
11	0.75	16.3	0.85	532.0	63.57	0.96	0.2	19845.0	76.64	**Quercetin**

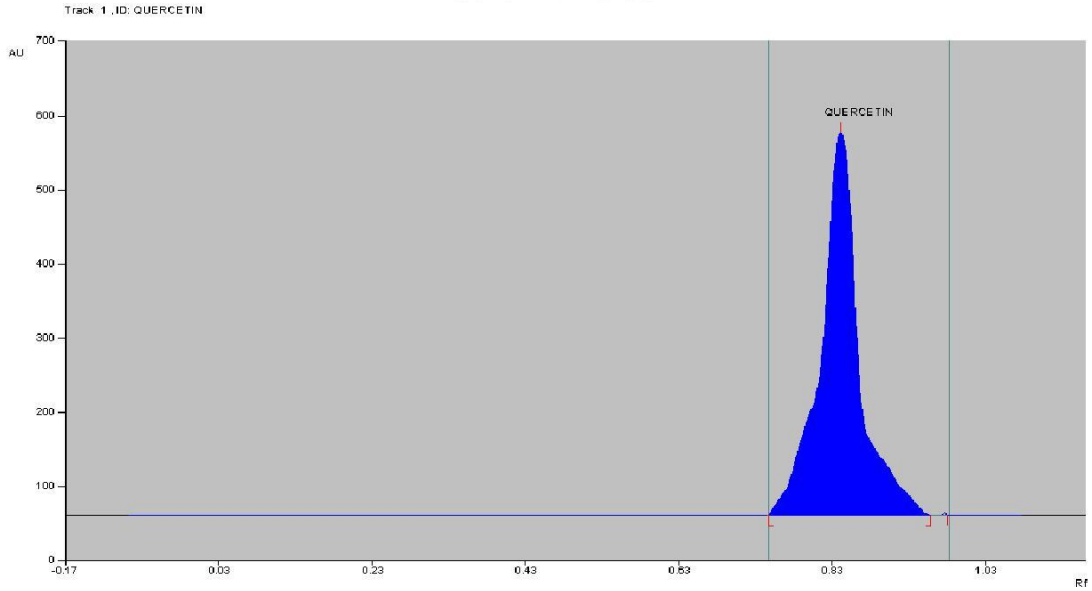

Fig. 2: Observation of Rf values and % area of the chromatogram of Quercetin at 254nm (std).

Table No 4: Observation of Rf values and %area of the chromatogram of ethanolic seeds extract of *Phoenix dactylifera* L

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	7.4	0.01	198.7	27.84	0.04	140.3	4766.7	23.36	Unknown*
2	0.04	140.6	0.05	151.0	21.15	0.10	65.3	4134.6	20.27	Unknown*
3	0.11	65.2	0.12	76.3	10.69	0.15	66.2	1879.1	9.21	**Rutin**
4	0.15	66.7	0.19	101.1	14.16	0.25	25.0	4180.9	20.49	Unknown*
5	0.26	24.2	0.30	34.4	4.82	0.36	10.9	1395.4	6.84	Unknown*
6	0.41	7.6	0.45	38.7	5.43	0.52	2.5	1295.3	6.35	Unknown*
7	0.80	0.0	0.84	44.4	6.22	0.86	32.3	1019.6	5.00	**Quercetin**
8	0.86	32.5	0.89	52.2	7.31	0.92	11.7	1274.6	6.25	Unknown*
9	0.92	11.8	0.94	17.0	2.38	0.98	0.6	456.0	2.23	Unknown*

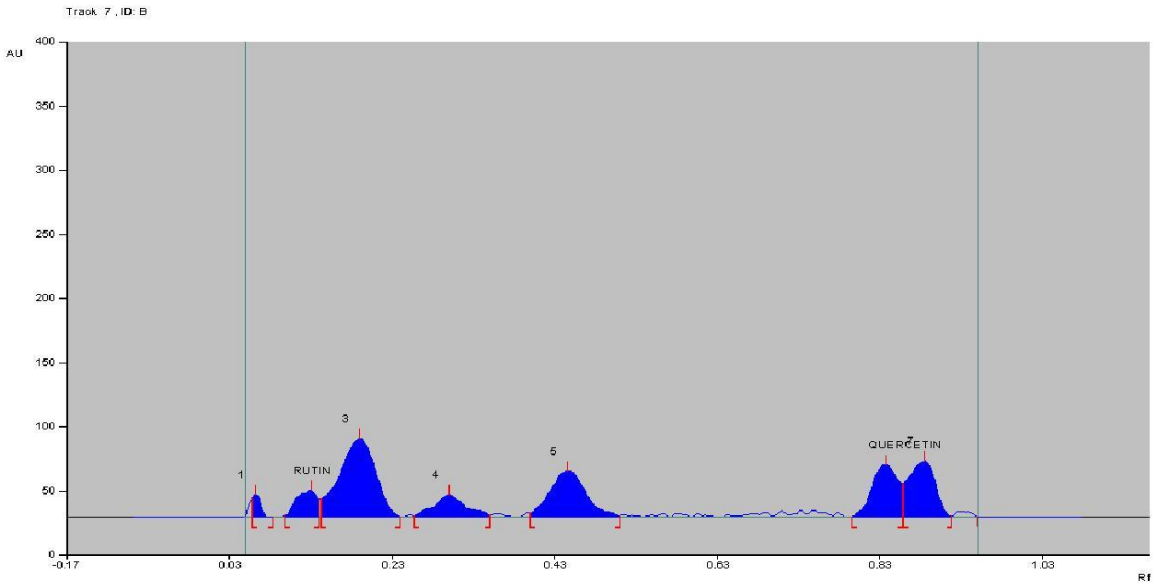

Fig. 3: Observation of Rf values and % area of the chromatogram of ethanolic seeds extract of *Phoenix dactylifera* L.

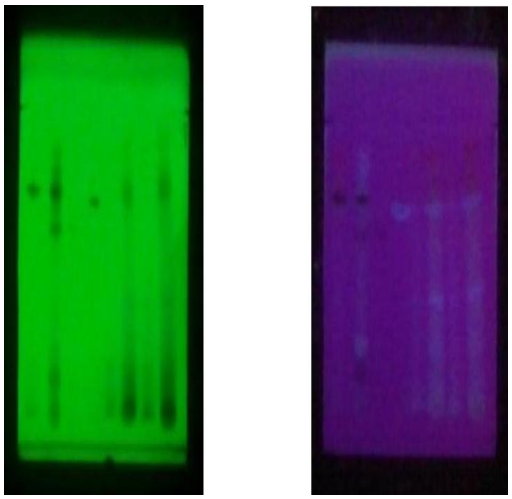

Fig. 4: Photo documentation of Standard and sample.

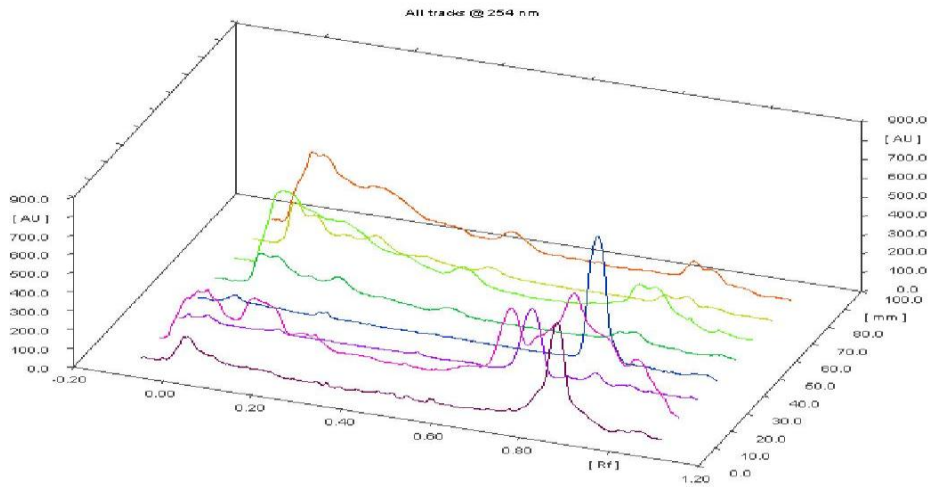

Fig. 5: 3D HPTLC Chromatography (Standard and seeds.

RESULTS AND DISCUSSION

MTT COLORIMETRIC ASSAY

Table 5: Effect of cyclophophamide (standard) on % cell survival of cell lines.

CELL LINES	CONCENTRATIONS (µg/ml)			
	10	20	40	80
HL-60	45.63	43.88	25.09	12.23
HT-29	50.92	48.02	35.89	24.09
A 549	53.11	49.25	41.26	28.26
A 431	56.03	51.26	41.88	31.45
MCF-7	57.99	53.09	42.06	37.65

All values are expressed as Mean±SEM. Data were analysed by One-way ANOVA followed by Dunnet's test.

Table 6: Effect of ethanolic seeds extract of *Phoenix dactylifera* L. on % cell survival of cell lines.

CELL LINES	CONCENTRATIONS (µg/ml)			
	10	20	40	80
HL-60	55.29	50.02	31.05	19.61
HT-29	61.56	60.76	52.06	42.71
A 549	63.36	61.03	55.99	51.71
A 431	67.49	63.72	60.38	53.74
MCF-7	77.32	77.28	74.42	65.68

All values are expressed as Mean±SEM. Data were analysed by One-way ANOVA followed by Dunnet's test.

SULPHORODAMINE B ASSAY

Table 7: Determination of Cytotoxicity by SRB assay.

Plant Extract	Conc. mg/ml	Hep 2			
		Absor-bance	% Inhibition	Ic50	R2
	10	0.032	130.56		
	5	0.069	110.53		
	2.5	0.0703	99.26		
	1.25	0.0852	88.35		
Phoenix dactylifera L.	0.625	0.0934	66.34	468	0.688
	0.312	0.10	43.65		
	0.156	0.103	36.45		
	0.078	0.125	23.12		
	0.0391	0.132	12.56		
	0.0196	0.134	10.23		

Cancer therapy in the form of surgery or radiotherapy is effective when the disease is early detected but many cancers are still diagnosed when cells from a primary tumor have already metastasized to other parts of the body and the main form of treatment at this point is chemotherapy.[16] Chemotherapy entails delivering drugs systemically so that they can reach and kill the tumor cells, but most of these drugs cause severe side effects in patients and, therefore, need to be used at suboptimal levels.[17] Plants have served as a rich source of therapeutic agents for many centuries, being used themselves or as the basis for synthetic drugs[18] and despite the great developments in organic synthesis, 55% of recent chemotherapeutic drugs are derived from or based upon natural products.[19] The use of plants as food and in folk and traditional medicine has made these natural resources one of the main agents in the research and development of cancer chemopreventive drugs.[20-22] The interest in alternative therapies using natural products is increasing, especially those derived from plants, due to the increasingly high number of cancer cases worldwide. The effect of RPMI 1640 media (control), cyclophosphamide (standard), ethanolic seeds extract of *Phoenix dactylifera* L on the growth of HL-60, HT-29, A 431, A 549 and MCF-7 cell lines were examined by MTT assay. Dose response curves constructed between the range of 10-80 ìg/ml, Results indicate that the antiproliferative effect strengthens with increase in the concentration of ethanolic seeds extract of *Phoenix dactylifera* L. Highest cytotoxicity was found against HL-60 and HT-29. However ethanolic extract have moderate activity on A 549, A 431 and MCF-7. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds, and that show *Phoenix dactylifera* L. effect on Hep 2 cell line upto 0.0196 mg/ml and that IC50 value on Hep 2 cell line was 468, *Phoenix dactylifera* L has no effect on normal healthy body cell So *Phoenix dactylifera* L second hand as antitumour action to diminish the side effect.

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