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In Vitro Screening Methods of Ruellia Patula Extract against Human Breast Cancer Cell Line

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ABSTRACT: The main goal of this study was evaluate the In vitro Screening methods of Ruellia patula Extract against Human Breast Cancer Cell Line MCF-7.The anticancer potential of cytotoxic extracts was determined by MTT assay. The ethanolic extract showed significant antiproliferative activities in a concentration and time dependent manner. The inhibitory concentration of extract was tested against target cell line and the results show in vitro leaf extract of Ruellia patula exhibited effective cytotoxic activity against MCF-7 and the inhibitory concentration (IC50) was recorded at 102.39µg/ml. The results of the present study conclude in vitro plant sample having more anticancer property.

Key words: Ruellia patula, Antiproliferative, MCF-7 cell line and MTT assay

I. INTRODUCTION:

Breast cancer is the most common cause of cancer in women and the second most common cause of cancer death in women in the U.S. Breast cancer refers to cancers originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Worldwide, breast cancer comprises 10.4% of all cancer incidences among women, making it the second most common type of non-skin cancer (after lung cancer) and the fifth most common cause of cancer death. In 2004, breast cancer caused 519,000 deaths worldwide (7% of cancer deaths; almost 1% of all deaths). Breast cancer is about 100 times more common in women than in men, although males tend to have poorer outcomes due to delays in diagnosis. Cancer cells are very similar to cells of the organism from which they originated and have similar (but not identical) DNA and RNA. [1- 4]

II. MATERIALS AND METHODS:

Collection of Plant:

The whole plant of Ruellia patula was collected from Valappady, Salem District. The leaves of the collected plants were separated and washed thoroughly in tap water in the laboratory to remove dust and shade dried in a well-ventilated place at room temperature. The dried leaves were ground to a coarse powder and subjected to solvent extraction.

Authentication of plant:

The plant was analysed, and the identity of the plant material was confirmed by a biological scientist, at Botanical survey of India, Coimbatore, India. The authentication number of the plant was BSI/SRC/5/23/2020/Tech.

Solvent extraction:

Ethanol was used as a solvent to prepare the plant extracts. The coarsely powdered leaves of the plant materials were direct soaked in 500 ml ethanol and then subjected to cold percolation for 14 days. The ethanol extract was concentrated by evaporating at a reduced pressure using a rotary evaporator. The extracts were subjected to a preliminary phytochemical analysis test for the identification of various phytochemical constituents as per the standard procedures.

LC-MS Analysis:

The LC-MS spectrums interpretation was performed using a spectrum. Database for organic

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compounds in SDBS application. Chromatography was performed according to Zakaria et al. where 20 µl of sample was injected onto a Agilent C18 reverse-phase column (4.0 × 250 mm, 1.8 µm) and held at 50°C with a constant flow rate of 0.4 ml/min and total LC run time of 30 min. Sample elution was performed in a gradient manner using mobile phase comprising of water containing 0.1% acetic acid (solvent A) and HPLC grade acetonitrile containing 0.1% acetic acid (solvent B). The mobile phase composition (A : B) was gradually increased from 5.95 to 15.85 over 25 min and returned to the initial condition (95%) for 5 min for solvent A and 5% to 85% for 25 min and then decreased to the initial condition (5 : 95) over the next 5 min. For the mass analysis, the source conditions were as follows: nebulizer pressure was 40 psi, drying gas flow was set at 12 L/min, and drying gas temperature was 350°C and the MS/MS acquisitions were performed in the negative and positive electrospray ionization mode, for the mass range of 150 to 1500 m/z. Data acquisition was performed by Agilent Mass Hunter workstation Data Acquisition, while data processing was carried out with Mass Hunter Qualitative Analysis software. In addition, MS/MS experiments were carried out in the automatic and multiple reaction monitoring (MRM) mode where automatic MS/MS low-energy collision dissociation (CID) was performed at 5–8 EV collision energy. Peak identification was carried out based on comparison with literature values and online databases. [5-7]

Cell Lines and Culture Medium

MCF-7 cell line was procured from NCCS, stock cell was cultured in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells were checked and centrifuged. Further 50,000 cells / well was seeded in a 96 well plate and incubated for 24 hrs at 37°C, 5% CO_2 incubator.

MTT Assay:

The cells were preincubated at a concentration 1.0 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations of test drug was added on to the partial monolayer in microtiter plates. The plate was then incubated at 37°C for 24hrs in 5% CO_2 atmosphere. After incubation the test solution in the wells were discarded and 100µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plate was incubated for 4h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100µl of DMSO was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. [8- 11]

IC_{50} :

The concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of compound) vs % cell inhibition. A line drawn from 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC_{50}, value. [11, 12]

Percentage inhibition of unknown extract against the two cell lines was calculated using the following formula:

$$\text{Absorbance of Test} = \frac{\text{Absorbance of Control} - \text{Absorbance of Standard}}{\text{Absorbance of Control}} \text{ X } 100$$

Where,
At = Absorbance of Test,
Ab = Absorbance of Blank (Media),
Ac= Absorbance of control (cells)
% cell inhibition = 100 − % cell survival

III. RESULTS:

Preliminary Phytochemical Analysis:

The Qualitative Phytochemical analysis was done by using of Ethanolic Extract. The results were shown in Table 1. It indicates the presence of Cardiac Glycosides, Flavanoids, Phenols, Steroids, Terpenoids and Proteins. The Extracted Plant materials were shown in Figure.1

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Sample	Extract
Alkaloids	-
Saponins	-
Tannins	-
Cardial glycosides	+
Flavonoids	+
Phenols	+
Steroids	+
Terpenoids	+
Quinones	-
Proteins	+

\+ Present - Absent

Table.1 Qualitative Phytochemical analysis of Ethanolic Extract

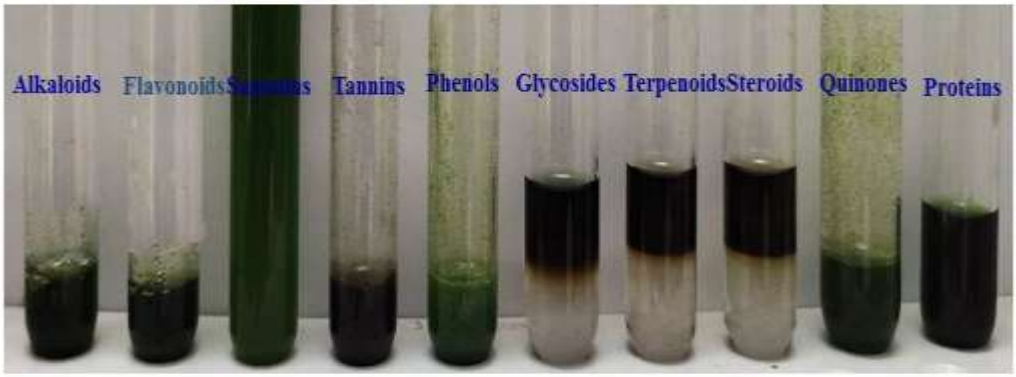

Figure.1 Ethanolic Extract of Phytochemical Compounds

Bioactive Compounds Identified in Ethanolic Extract by LC-MS Analysis:

Name of the compound	Retention time (min)	Molecular formula	Molecular weight
Oleanolic acid	22.93	C30 H48 O3	456.36
(9cis)-Retinal	16.34	C20 H28 O	284.21
Geniposidic acid	4.33	C16 H22 O10	374.12
Nootkatone	14.12	C15 H22 O	218.16
Maslinic acid	21.89	C30 H48 O4	472.35
19-Nortestosterone	19.72	C18 H26 O2	274.19
Bioside	6.28	C20 H30 O12	462.17
6-O-Methylscutellarin	13.88	C22 H20 O12	476.09
Baicalin	13.47	C21 H18 O11	446.08
Progesterone	19.22	C21 H30 O2	314.22
Scrophulein	18.25	C17 H14 O6	314.07
Andrographolide	17.02	C20 H30 O5	350.2
Cafestol	20.54	C20 H28 O3	316.2
Kaempferol-7-O-glucoside	12.52	C21 H20 O11	448.1
Ursolic acid	22.97	C30 H48 O3	456.36
D-(-)-Quinic acid	0.96	C7 H12 O6	192.06

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18-β-Glycyrrhetinic Acid	22.99	C30 H46 O4	470.33
Jasmonic acid	14.81	C12 H18 O3	210.12
Neosolaniol	11.45	C19 H26 O8	382.16
Ostruthin	19.38	C19 H22 O3	298.15
Proscillaridin A	20.65	C30 H42 O8	530.28
Etiocholanolone	20.21	C19 H30 O2	290.22
Caffeic acid	19.98	C9 H8 O4	180.04

Table.2 Spectral Analysis of Bioactive Compound by LC- MS Method

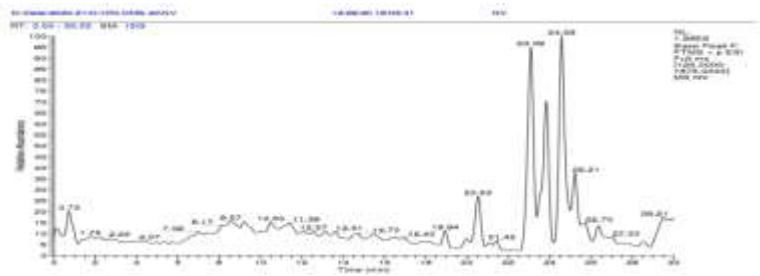

Figure.2 Spectral Analysis of Bioactive Compound by LC- MS Method

In- vitro Study-MTT Assay:

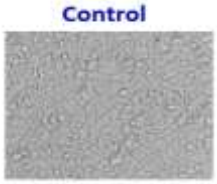

Figure 3. Control/ Blank cell line

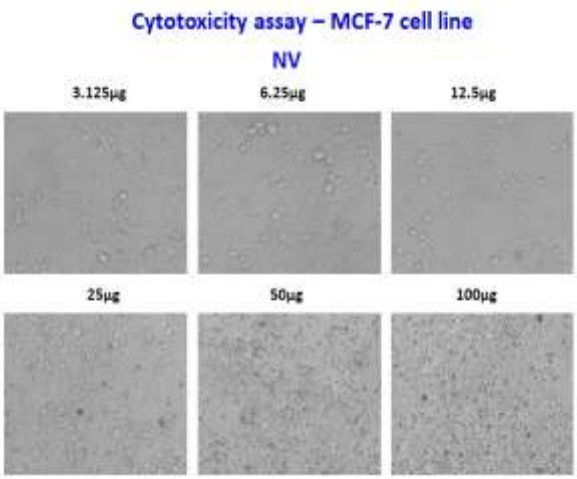

Figure.4 MCF-7 Cell Line of Extract

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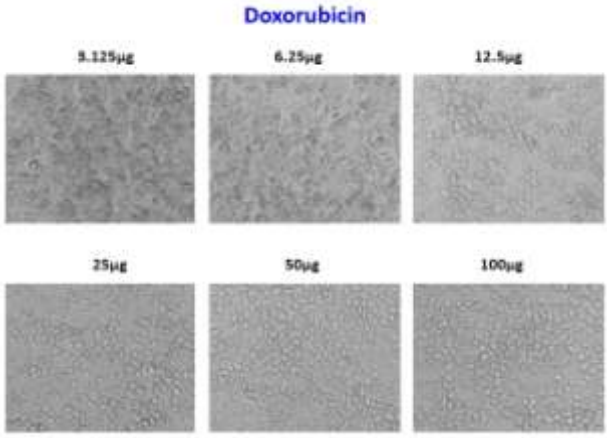

Figure.5 MCF-7 Cell Line – Doxorubicin

MCF-7 (Human Breast adenocarcinoma) cell line was procured from NCCS, stock cell was cultured in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells were checked and centrifuged. Further 50,000 cells / well was seeded in a 96 well plate and incubated for 24 hrs at 37°C, 5% CO_2 incubator.

IC 50 Calculations - MTT assay - MCF-7 cell line:

MTT assay - MCF-7 cell line								
Concentration Unit: µG								
NV	BLANK	UNTREATED	3.125	6.25	12.5	25	50	100
Reading 1	0.029	1.24	1.214	1.107	1.007	0.93	0.767	0.598
Reading 2	0.03	1.233	1.204	1.116	1.015	0.922	0.769	0.592
Reading 3	0.03	1.238	1.209	1.11	1.003	0.927	0.764	0.596
Mean	0.0297	1.237	1.209	1.111	1.0083333	0.9263333	0.7666667	0.59533
Mean OD-Mean B		1.2073333	1.1793333	1.0813333	0.9786667	0.8966667	0.737	0.56567
Standard Deviation		0.0036056	0.005	0.0045826	0.0061101	0.0040415	0.0025166	0.00306
Viability %		100	97.680839	89.563777	81.060188	74.26836	61.043622	46.8526
							IC 50 VALUE = 102.39 µg	

Table.3 Percentage Cell Viability of Ruellia patula

Doxorubicin	BLANK	UNTREATED	3.125	6.25	12.5	25	50	100
Reading 1	0.029	1.24	0.752	0.437	0.119	0.037	0.03	0.03
Reading 2	0.03	1.233	0.747	0.437	0.125	0.036	0.034	0.03
Reading 3	0.03	1.238	0.748	0.43	0.118	0.035	0.031	0.031
Mean	0.0297	1.237	0.749	0.4346667	0.1206667	0.036	0.0316667	0.03033
Mean OD-Mean B		1.2073333	0.7193333	0.405	0.091	0.0063333	0.002	0.00067
Standard Deviation		0.0036056	0.0026458	0.0040415	0.0037859	0.001	0.0020817	0.00058
Viability %		100	59.580342	33.545003	7.5372722	0.5245721	0.1656543	0.05522
							IC 50 VALUE = 2.43 µg	

Table.4 Percentage Cell Viability of DOXORUBICIN

International Journal of Advances in Engineering and Management (IJAEM)
Volume 6, Issue 07 July 2024, pp: 243-249 www.ijaem.net ISSN: 2395-5252

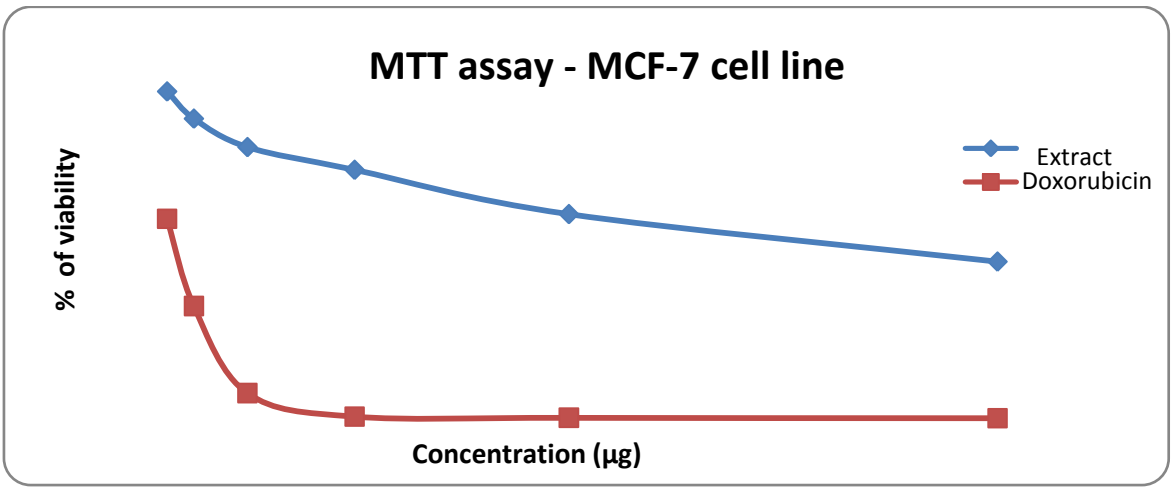

Figure.6 MTT Assay – MCF-7 Cell line

IV. DISCUSSION:

The preliminary phytochemical screening of the ethanol extracts of all the plants revealed the presence of alkaloid, flavonoid, saponin, steroid, phenol, tannin, glycoside and terpenoid (Table 1). The effect of ethanol extract of Ruellia patula and doxorubicin (standard) on the growth of MCF-7 cell lines was examined by the MTT assay. The main aim is to evaluate the In vitro anticancer activity of ethanolic extract of Ruellia patula leaves on the murin mastocytoma cancer cell line (P815), and the phytochemical analysis of the extract as well. The ethanol extract was obtained by traditional method adapted to laboratory conditions and the phytochemical analysis was based on differential staining and precipitation reactions. In vitro anticancer effect was evaluated by the cellular cytotoxicity against the murin mastocytoma cell line (P815). Cellular cytotoxicity was determined by the MTT assay. Phytochemical screening of aqueous extract showed several chemical groups: alkaloids, flavonoids (flavones), tannins, coumarins, sterol, triterpenoids, saponins and reducing compounds. The in vitro anticancer effect, showed a dose dependant cytotoxic effect. It was observed, that the maximum activity of the extract at the highest concentration tested (100µg/ml) was 50% (±1.5). Further on lower doses of 3.12; 6.25; 12.15; 25 50 and 100 µg/ml. Percent growth inhibition observed by the extract was between 43 and 47 %, the IC50 value of the extract (100µg/ml,. Our results suggest that ethanolic extract of Ruellia patula leaves contains several chemical groups and possess a weak in vitro anticancer effect against P815 tumor cell line. The cytotoxic or antiproliferative activity of Ruellia patula extracts may be mediated by their terpene. The phytocompounds found in the in vivo leaf and in vitro callus ethanol extracts using LC-MS analysis Table.2 the identified phytocompounds are tabulated in Table 2. The LC-MS chromatogram of both leaf extracts is presented in, Figure.2 with the retention time and intensity of the compounds. The identified compounds of in vivo leaf and in vitro callus ethanol extracts were based on their structure and molecular mass with the degree of similarity in vivo leaf ethanol extract have a total of nine compounds in 30 min elustion, the first eluted compound was Kaempferol which also blocks the cell cycle and ER signalling acting. Doses of 50-100 µm decreased the cell viability in MCF7.Dose dependent response were obtained between the range 50-100 µg/ml for plant extracts and doxorubicin (standard-control), expenses decreasing number of viable cells with increasing concentration of plant extracts as well as doxorubicin. Calculation of IC_{50} value was done using Graph pad Prism Software (ver.5.01). The susceptibility of cells to the different plant extracts and doxorubicin was characterized by IC_{50} and R2 values (Tables 3and 4). An IC_{50} value of MCF -7 cell line was 102.39, 2.43 µg/ml NV and doxorubicin respectively. The IC_{50} values of extract on cell line less than 100µg/ml is categorized as a potential cytotoxic substance ·Doxorubicin extract treatment on cancer cell lines showed significant decrease in growth rate compared with control. On the other hand the percentage of non-viable cell lines increased with the increasing concentration of extracts. These results were in concordance with the studies investigated on the cytotoxic effect of Goniothalamin towards human breast cancer cells.

V. CONCLUSION:

The evaluation of Ruellia patula leaves extract confirmed the existence of various phytochemicals like Cardiac Glycosides, Flavanoids, Phenols, Steroids, Terpenoids and Proteins. They were characterized by LC-MS analysis. Ruellia patula leaves extract presents anti-proliferative activity towards breast adenocarcinoma MCF-7 cell lines by producing cell death through morphological changes, like apoptosis of cell. This cytotoxicity showed selectivity towards the proliferation of breast cancer cells, indicating selective antitumor properties in Ruellia patula extract against tumor cells. The extract exhibited effective cytotoxic activity against MCF-7 and the inhibitory concentration (IC50) was recorded at 102.39µg/ml. The in vivo studies clearly indicated that the extract has shown moderate capacity to inhibit the growth of tumor in breast carcinoma. Moreover, the extract studied on breast cancer cell lines MCF-7 with different concentrations of showed a mean reduction of cell viability for a concentration of 100 µg/ml.

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